Investigating the effects of three herbicides - Kamba, 2,4-D and Roundup on *Salmonella enteric* serovar *Typhimurium* growth and antibiotic tolerance phenotypes

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Abstract

Herbicides are a common tool in weed control. With the introduction of genetically modified herbicide-tolerant crops, there has been a dramatic increase in the use of particular herbicides. Herbicides contaminate the environment and food and feed and can come into contact with non-target organisms, especially bacteria. S*almonella enteric* serovar *Typhimurium*, which is a human and animal pathogen, was chosen to investigate if the commercial formulations of three herbicides – Kamba, 2,4-D and Roundup are toxic to bacteria and whether sub-lethal concentrations cause a response to antibiotics. In addition, earlier work demonstrating an effect of salicylic acid on antibiotic response was reconfirmed in this study.

The herbicides were toxic to *S. typhimurium* at concentrations above the manufacturers recommended application rates. A key finding of this study was that when *S. typhimurium* was grown in sub-lethal concentrations of the herbicides, it demonstrated a change in its susceptibility to various antibiotics. Kamba and 2,4-D caused increased *tolerance* of chloramphenicol, tetracycline, ampicillin and ciprofloxacin and increased *sensitivity* to kanamycin. Exposure to Roundup caused increased *sensitivity* to chloramphenicol and tetracycline and increased *tolerance* towards kanamycin and ciprofloxacin. Roundup had no measureable affect on ampicillin susceptibility.

The minimum concentrations of herbicides that induced an antibiotic response were within the recommended application rates. Furthermore, the minimum 2,4-D concentration that induced tetracycline, chloramphenicol and ampicillin tolerance was at or below the maximum residue limits set for food and feed commodities. Simultaneous exposure to an herbicide and an antibiotic was necessary for the induction of antibiotic tolerance. In addition, the effect of the herbicide on the antibiotic response was faster than the lethal effect of the antibiotics. Kamba-induced chloramphenicol, tetracycline, ampicillin and ciprofloxacin tolerance was maintained in the absence of Kamba once tolerance was induced by simultaneous exposure to Kamba and antibiotic.

The emergence of antibiotic tolerance is an important health issue that may compromise treatment of serious bacterial infections. The widespread use of herbicides in agricultural, urban and domestic settings increases the number of bacteria that are exposed to herbicides. The tolerance induced by the herbicides may increase the frequency of antibiotic tolerant strains, increase the chance of co-exposure to antibiotics, and increase the potential for failure to treat bacterial infections as a result.

Abbreviations

ae	acid equivalent
Amp	ampicillin
AMPA	aminomethylphosphonic acid
C	centigrade
cDNA	complementary deoxyribonucleic acid
cfu	colony forming units
Cip	ciprofloxacin
CM	chloramphenicol
DNA	deoxyribonucleic acid
EOP	efficiency of plating
EPA	Environmental Protection Agency
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
Fig.	figure
g	g-force
gal	gallons
GM	genetically modified
lbs	pounds
LB	luria broth
M	moles/liter
MCL	maximum contamination level
MDR	multidrug resistant
mg	milligrams
MIC	minimum inhibitory concentration
ml	milliliters
MRL	maximum residue limit

mRNA	messenger ribonucleic acid
nm	nanometers
OD_{600}	optical density
PAβN	Phenylalanine-arginine β-naphthylamide
ppb	parts per billion
ppm	parts per million
qPCR	real-time quantitative polymerase chain reaction
RNA	ribonucleic acid
RND	Resistance-nodulation-cell division
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SEM	standard error of mean
Tet	tetracycline
tRNA	transfer ribonucleic acid
µl	microliters
µm	micrometers
U.K.	United Kingdom
U.S.A.	United States of America
WHO	World Health Organization

Chapter One

1. Introduction

The development of glyphosate-tolerant crops has given an added advantage to the agricultural industry by making weed control simpler and more flexible than is available for conventional crops (Duke & Powles, 2008). The rapid adoption of glyphosate-tolerant soybean, corn and cotton has made glyphosate the world's best selling agrochemical (Baylis, 2000; Nandula *et al.*, 2005; Service, 2007). In the United States, the adoption of genetically modified soybean and cotton reached 94% by 2011 and 2012, respectively (Beckie, 2011; Heinemann *et al.*, 2014). Agrochemical companies are in the process of developing and commercialising crops tolerant to dicamba and 2,4-D in addition to glyphosate (Benbrook, 2012; Crespo *et al.*, 2013). Despite the usefulness of herbicides in controlling weeds, they may pose a risk to non-targeted organisms in the environment (Botelho *et al.*, 2012). A number of studies have researched the toxic effects of herbicides (Williams *et al.*, 2000; Bukowska, 2006; Gonzalez *et al.*, 2006; Gasnier *et al.*, 2009). However, there is limited research on the effects herbicides have on bacteria, especially sub-lethal effects.

The widespread use and sometimes misuse of antibiotics has led to the emergence and spread of antibiotic-tolerant bacteria, creating a global threat to both human and animal health (Tomson & Vlad, 2014). Increased antibiotic tolerance in pathogenic bacteria can lead to treatment failure (Fernandez *et al.*, 2011). Due to the slow rate of development and introduction of new antibiotics, any increase in tolerance is a serious risk to the success of the currently available antibiotics (Mishra *et al.*, 2012). There are a number of chemicals that can cause an increase in antibiotic tolerance and they do so through different mechanisms. Salicylic acid is a chemical that is known to induce a response in *Escherichia coli* and other bacteria to various antibiotics (Price *et al.*, 2000). It reduces the accumulation of antibiotics within the cell by changing the influx and efflux of antibiotics (Price *et al.*, 2000). This allows the bacterial cell to survive higher amounts of antibiotics (Randall & Woodward, 2001). The structures of 2,4-D

and dicamba are similar to that of salicylic acid, therefore we hypothesized that the effects of these herbicides could extend beyond toxicity to antibiotic tolerance phenotypes. With the wide spread use of glyphosate, 2,4-D and dicamba and the recurrent contamination of the environment by these herbicides, it is vital to have a solid understanding of any adverse effects herbicides have on bacteria.

1.1 Herbicides

Glyphosate [*N*-(phosphonomethyl)glycine] first introduced in 1974 (Powles, 2008; Beckie, 2011), is a non-selective, broad-spectrum, post-emergence herbicide and is used in a number of countries (Nandula *et al.*, 2005; Powles, 2008). This 'once-in-a-century' herbicide is used in a wide variety of environments including urban areas, national parks and crop farms (Powles, 2008; Beckie, 2011). 2,4-dichlorophenoxyacetic acid (2,4-D) and 3,6-dichloro-2-methoxybenzoic acid (dicamba) are both synthetic auxin herbicides developed in 1940 and 1960 respectively and are also used worldwide (Green, 2014). Both these herbicides are used to control a wide range of dicotyledonous weeds in cereal crops and pastures in combination with glyphosate in no-tillage systems (Zabaloy *et al.*, 2010; Cao *et al.*, 2011).

1.1.1 Mode of action of dicamba, 2,4-D and glyphosate

Dicamba and 2,4-D are structurally similar to and mimic the natural auxin indole-3-acetic acid in plants (Gleason *et al.*, 2011; Mithila *et al.*, 2011). Auxins are plant hormones that regulate plant growth and development (Dharmasiri *et al.*, 2005). The exact mode of action of auxinic herbicides remains unclear (Mithila *et al.*, 2011). However, the effects of auxin herbicides can be divided into three parts: abnormal growth and gene expression, inhibition of growth and physiological responses, and finally cell death (Gleason *et al.*, 2011). During the first phase, there is a rapid increase in ethylene and abscisic acid, which cause the stomata to close. This is followed by abnormal growth and tissue swelling (Grossmann, 2010). In the second phase, growth of the root and shoot are inhibited (Bukowska, 2006) and the accumulation of abscisic acid reduces the formation of carbon dioxide and increases the accumulation of hydrogen peroxide within the plant (Grossmann, 2010). In the final phase, the accumulation of hydrogen

peroxide may cause damage to plant tissue by destroying the membrane integrity which leads to death (Grossmann, 2010; Gleason *et al.*, 2011).

Glyphosate inhibits the enzyme 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) by binding to it, forming a complex (Sammons & Gaines, 2014). EPSPS catalyzes the formation of 5-enolpyruvylshikimate 3-phosphate in the shikimate pathway. The inhibition of EPSPS reduces the feedback inhibition of the shikimate pathway (Duke & Powles, 2008). This causes the accumulation of shikimic acid and other precursors of the chrismate pathway that are necessary for the synthesis of three aromatic amino acids (phenylalanine, tryptophan, and tyrosine) (Nandula *et al.*, 2005; Service, 2007; Duke & Powles, 2008; Gao *et al.*, 2014). The exact mechanism that kills the plant is not clear (Gao *et al.*, 2014). However, reductions in the amino acids cause several metabolic disturbances, such as inhibition of protein and secondary product synthesis and may also make the plant susceptible to various plant pathogens (Johal & Huber, 2009). This may cause the plant to wither and die (Nandula *et al.*, 2005; Service, 2007). The shikimic acid pathway is not limited to plants. It is also found in bacteria and fungi (Busse *et al.*, 2001) and it has been shown that the commercial formulation of glyphosate (Roundup) inhibits growth of food microbes (Clair *et al.*, 2012).

1.1.2 Glyphosate-tolerant crops

Glyphosate-tolerant soybean, cotton and corn were introduced in 1996, 1997 and 1998 respectively, by Monsanto under the trade name Roundup Ready ® (Castle *et al.*, 2006; Young, 2006; Givens *et al.*, 2009). Crops were made tolerant by inserting a homologue of the EPSPS from *Agrobacterium sp.* This transgenic enzyme functions at higher levels of glyphosate and allows the function of the shikimate pathway by maintaining the normal aromatic amino acid levels (Nandula *et al.*, 2005; Dill *et al.*, 2008). This allowed farmers to apply glyphosate over a wide time period to tolerant crops in order to eliminate weeds with negligible crop damage (Powles, 2008).

Although glyphosate-tolerant plants are able to withstand high glyphosate concentrations, they may still be subject to unintended negative effects caused by the herbicide. For example, the application of glyphosate on glyphosate-tolerant soybeans has been found to alter the

polyunsaturated fatty acid content of seeds, decrease photosynthesis rates and nutrient content in leaves and seeds (Zobiole *et al.*, 2010). The application of glyphosate may also increase the vulnerability of plants to fungal diseases because there is an increase in the level of fungal pathogens following glyphosate application (Johal & Huber, 2009).

On the other hand, the introduction of glyphosate-tolerant crops offered improved weed control with reduced labour inputs, time, herbicide costs and enabled farmers to use glyphosate in ways that were previously impossible (Castle *et al.*, 2006; Young, 2006; Beckie, 2011; Benbrook, 2012). Since the introduction of glyphosate-tolerant crops, there has been a steady increase in the adoption of these crops worldwide (Dill *et al.*, 2008; Green, 2012). Almost the entire soybean crop is glyphosate-tolerant (99%) in Argentina (Powles, 2008).

Instead of integrating glyphosate into weed management utilizing multiple herbicides, growers have relied mainly on glyphosate (Young, 2006). This has led to a dramatic increase in the use of glyphosate and subsequently, a decrease in the use of other herbicides, resulting in a monoculture of herbicides (Powles, 2008). Before the introduction of herbicide-tolerant crops, the dominant herbicides used on corn, cotton and soybean were atrazine, toxaphene and chloramben, respectively. However, since then the dominant herbicide used for all three crops is glyphosate in at least some agroecosystems (Table 1.1).

Table 1.1. A comparison of dominant pesticide use on crops pre and post herbicide-tolerant crops

Crop	Pre-Herbicide Tolerant Crops (1968)	Post-Herbicide Tolerant Crops (2008)
Corn	Atrazine (57%)	Glyphosate (33%)
Cotton	Toxaphene (31%)	Glyphosate (37%)
Soybean	Chloramben (34%)	Glyphosate (85%)

Dominant herbicide and the percentage of total pounds of active ingredient applied in the United States. Source: United States Department of Agriculture – Pesticide use in U.S. Agriculture, May 2014 (Fernandez-Cornejo *et al.*, 2014)

In the U.S., not only has there been a dramatic increase in glyphosate use, but the area treated with glyphosate has also increased (Table 1.2). Prior to the development of herbicide-tolerant crops, only 21% of the area planted with soybeans was treated with glyphosate and this increased to 90% following the adoption of herbicide-tolerant crops. The number of

applications has also increased (Table 1.2). The largest increase was for cotton crops (an increase to 2.4 applications per year). The increase in annual glyphosate applications may be due to the additional applications made after crop emergence (Young, 2006). The average concentration of active ingredient in each application has also increased, for example, the average amount of glyphosate in each application used for soybeans was 0.57 Ib/application/acreage in 1995 and by 2006 it increased to 0.81 Ib/application/ acreage (Table 1.2).

Pesticide use on corn, cotton and soybeans in the U.S. has not been tested or summarized since 2005 and 2006. Since then there may have been changes in glyphosate use. Benbrook (2012) estimates that there has been an increase of 12.9% in total glyphosate use between 2005 and 2011 for corn and an increase of 3.3% between 2007 and 2011 for soybean (Benbrook, 2012). In contrast, glyphosate use on cotton crops was projected to decrease between 2007 and 2010, which may be due to increased farmer knowledge about glyphosate-tolerant weeds, and improved management strategies (Benbrook, 2012).

Table 1.2. Trends of glyphosate usage pre and post herbicide-tolerant crops

Crop	Pre-Herbicide Tolerant Crops	Post-Herbicide Tolerant Crops
	Average Total Active Ingredient Applied per Crop Year (Ib/ac)	
Corn	152.1 (1995)	1208.8 (2005)
Cotton	172.3 (1995)	1567.9 (2005)
Soybean	451.3 (1995)	4679.2 (2006)
	Average Percentage of Acreage treated with Glyphosate (%)	
Corn	11% (1995)	40% (2005)
Cotton	15% (1995)	82% (2005)
Soybean	21% (1995)	90% (2006)
	Average Number of Glyphosate Applications per Year	
Corn	1 (1995)	1.3 (2005)
Cotton	1.1 (1995)	2.4 (2005)
Soybean	1 (1995)	1.7 (2006)
	Average Active Ingredient per Application (Ib/appli/ac)	
Corn	0.78 (1995)	0.75 (2005)
Cotton	0.57 (1995)	0.70 (2005)
Soybean	0.57 (1995)	0.81 (2006)

Data adapted from the United States National Agricultural Statistics Service (NASS); Agricultural Chemical Use Database (online). Available at http://www.pestmanagement .info/nass/ (verified 20/6/2014). Number in parentheses indicates the year data was collected.

Although there has been an increase in glyphosate use, many authors suggest that the total amount of pesticidal active ingredients applied to genetically engineered crops has decreased compared to conventional crops (Kleter *et al.*, 2007; Wright *et al.*, 2010). This may be due to the reduction of other insecticides and herbicides (Kleter *et al.*, 2007; Wright *et al.*, 2010). However, a study that quantified the pesticide use in the U.S. over 16 years found that there was an overall increase in the amount of pesticide used (Benbrook, 2012). This study differentiated the changes in herbicide and insecticide use. Results showed that between 1996 and 2011, there was an increase in herbicide use (239 million kilograms) while insecticide use reduced by 56 million kilograms. Overall, pesticide use increased by 183 million kilograms (Benbrook, 2012).

1.1.3 Emergence of glyphosate-tolerant weeds

The increase in glyphosate use and insufficient diversity in weed management has created a tremendous selection pressure for glyphosate-tolerant weeds (Powles, 2008). This caused a

shift in weed species to plants that are tolerant to glyphosate, including unwanted Roundup Ready crop plants (Nandula *et al.*, 2005; Young, 2006; Powles, 2008). There were no known glyphosate-tolerant weeds prior to the introduction of glyphosate-tolerant crops (Powles, 2008) and it was thought that tolerance to glyphosate was unlikely to evolve (Bradshaw *et al.*, 1997; Benbrook, 2012; Ghanizadeh *et al.*, 2013). However, tolerant weeds have now been reported in a number of countries and attributed to overuse of glyphosate (Beckie, 2011).

In the year 2000, the first glyphosate-tolerant horseweed was reported in a field planted with glyphosate-tolerant soybean in the U.S., which exhibited up to 13-fold more glyphosate tolerance (VanGessel, 2001). Since 2000, there have been 28 glyphosate-tolerant species reported across a number of states in the U.S. and across the world (Ghanizadeh *et al.*, 2013; Heap, 2014b). The increase in glyphosate-tolerant weeds is becoming problematic as it reduces the efficiency and sustainability of glyphosate (Salas *et al.*, 2012).

Mechanisms that cause plants to develop tolerance include: alterations in the binding site of glyphosate, reduced translocation into the plant (Ghanizadeh *et al.*, 2013), and reduced herbicide absorption (Nandula *et al.*, 2005; Beckie, 2011). For some weed species, tolerance has been linked to increased amplification, expression and enzymatic activity of EPSPS (Gaines *et al.*, 2011; Salas *et al.*, 2012).

1.1.4 Herbicide residues in the environment

The widespread use of herbicides in large volumes can cause pollution in the neighbouring environments as they have the potential to move away from the application area and accumulate elsewhere (Chang *et al.*, 2011). Glyphosate is water soluble so it is not surprising that a significant amount of glyphosate may leach through surface and subsurface runoff into aquatic environments (Borggaard & Gimsing, 2008; Lane *et al.*, 2012). Similarly, dicamba is highly soluble in water but it does not bind well to soil particles, which gives it the potential to leach at a higher rate (Cojocaru *et al.*, 2013). A study on Brazilian soils found that dicamba was one of the least absorbed herbicides (Oliveira *et al.*, 2001). 2,4-D is moderately soluble in water and is weakly absorbed by soil particles, increasing the threat of surface and ground water contamination (Hermosin *et al.*, 2006).

The pesticide leaching potential index is used to estimate the potential of an herbicide to leach. This index is calculated by taking into account the half-life of the pesticide, the rate of application, the fraction of herbicide that comes into contact with the soil during application and the affinity of the herbicide for soil organic matter (McLaughlin *et al.*, 1994). The higher the number, the more likely that the herbicide will move through soil and potentially contaminate surface or ground water (Entry & Sojka, 2012). Of the three herbicides, dicamba has the highest pesticide leaching potential value (58 to 74) followed by 2,4-D (45) and glyphosate (23) (McLaughlin *et al.*, 1994).

Glyphosate, dicamba and 2,4-D have been found to contaminate soil, groundwater and air (Shin *et al.*, 2011). A study on the levels of glyphosate in air and rain fall in Mississippi and Iowa found that 60% to 100% of the samples collected between 2007 and 2008 had glyphosate residues, although at low concentrations (< 0.1 ppm) (Chang *et al.*, 2011). Glyphosate and its main degradation product, aminomethylphosphonic aicd (AMPA) have also been found in soils that are frequently treated with glyphosate (Kremer & Means, 2009) and in surface and ground waters (Van Stempvoort *et al.*, 2014). In a study that summarized glyphosate occurrence over 10 years in the U.S., glyphosate was detected in streams (52%), rivers (53%) and lakes and ponds (34%) (Battaglin *et al.*, 2014).

The detection of glyphosate residues is not limited to soil, air and water, but has also been found in food. In 2010, glyphosate residues were found in 5.6% of the tested bread samples in the U.K. at concentrations as high as 0.5 ppm (Benbrook, 2012). A recent study which compared the amount of glyphosate residues in conventional, organic and glyphosate-tolerant soybeans, found that soybeans that were glyphosate-tolerant contained high levels of glyphosate (3.3 ppm) (Bohn *et al.*, 2014). On the other hand, soybeans that were organic or treated using a conventional method did not contain glyphosate (Bohn *et al.*, 2014).

A survey of drinking water reservoirs in North America found 2,4-D and dicamba in 100% and 86% of the samples, however, none were above the Canadian drinking water guidelines (Donald & Cessna, 2007). Similarly, these herbicides have been detected in farm runoff and in aquatic environments that are in close proximity to farms (Kuo *et al.*, 2012). Dicamba and 2,4-D were

detected in rainfall in Canada at concentrations that were occasionally above the Canadian Drinking Water Guidelines (Filkowski *et al.*, 2003). Herbicide residues in the environment are not only from agricultural use; but also urban herbicide use (Ensminger *et al.*, 2013). Dicamba and 2,4-D were the most frequently detected herbicides in urban waterways that were unaffected by agricultural inputs (Ensminger *et al.*, 2013). Herbicide residues can also be present in homes, especially in carpet dust (Colt *et al.*, 2004). Up to 1.5 ppm 2,4-D and 0.037 ppm dicamba have been found in carpet dust across a number of states in the U.S. (Colt *et al.*, 2004).

Manufacturers generally recommend herbicide doses high enough to ensure effective weed control of a broad range of species and to prevent the appearance of tolerant weeds (Crespo *et al.*, 2013). Due to the potential of these herbicides leaching into the environment, national governments and international agencies have established Maximum Residue Limits (MRLs) which are the maximum concentrations legally allowed in food or feed commodities (MacLachlan & Hamilton, 2010; Shin *et al.*, 2011). MRLs are determined under supervised trials and are set so that any adverse side effect to human health and the environment are kept to a minimum (MacLachlan & Hamilton, 2010; Horvath *et al.*, 2014).

Under the current practice for determining MRLs, the number of field trials that are used are dependent on the amount of crop grown and its importance in the diet or trade (MacLachlan & Hamilton, 2010). For example, in Australia major crops require a minimum of 6 to 8 trials while minor crops require only 3 to 4 trials (MacLachlan & Hamilton, 2010). A new study found that a small number of trials (3 to 5) may compromise the accuracy of the estimated MRLs because the dietary intake of herbicides by consumers may be underestimated (Horvath *et al.*, 2014). Hence, the level of herbicide exposure for humans and animals may be higher, which may lead to adverse effects. In addition, the emergence of glyphosate-tolerant weeds is prompting farmers to use higher rates of application and more often (Green, 2014). This may further increase the accumulation of herbicide residues in the environment.

1.1.5 Effects of herbicides on non-target organisms

Herbicides do not target only weeds (Bukowska, 2006). The herbicides that are detected in the environment may affect non-target organisms in a number of ways, including decreased growth rate, changes in behavior or even death (Bukowska, 2006). In recent years, there has been increased concern regarding the safety of herbicides. There are conflicting opinions regarding the safety of dicamba, 2,4-D and glyphosate. Nevertheless, agrochemical companies and a number of regulatory agencies consider herbicides to be environmentally safe (Munro *et al.*, 1992; Dill, 2005; Behrens *et al.*, 2007; Shehata *et al.*, 2013). However, a number of studies have reported possible side effects of these herbicides on non-target organisms.

Many studies have shown the genotoxicity potential of 2,4-D and dicamba. Filkowski *et al.* (2003) showed that both these herbicides caused a concentration-dependent increase in homologous recombination and A to G point mutations in *Arabidopsis thaliana*. Only 0.1 ppm 2,4-D and 0.12 ppm dicamba were sufficient to increase the frequency of A to G mutations by 49.5% and 65%, respectively (Filkowski *et al.*, 2003). 2,4-D, dicamba and a commercial formulation of dicamba significantly increased the frequency of sister chromatid exchange in: mice that were treated with 100 to 200 ppm 2,4-D (Madrigal-Bujaidar *et al.*, 2001), mammal cells treated with 200 to 500 ppm dicamba (Gonzalez *et al.*, 2006) and Chinese hamster ovary cells treated with 1 ppm to 500 ppm of either dicamba or Banvel® (commercial formulation of dicamba) (González *et al.*, 2007). Furthermore, 2,4-D was shown to increase the somatic mutation and recombination in *Drosophila* at 221 ppm (Bukowska, 2006). In addition, a commercial formulation of 2,4-D significantly increased chromatid and chromosome breaks in human lymphocytes at 0.4 ppm and 4 ppm (Zeljezic & Garaj-Vrhovac, 2004).

2,4-D has also been shown to affect soil organisms. The growth of earthworms in soil were inhibited at 500 to 1000 ppm 2,4-D (Correia & Moreira, 2010). *E. coli* growth was inhibited when incubated in media containing 2,4-D (between 442 and 884 ppm) and at sub-inhibitory concentrations 2,4-D affected the protein content (Balague *et al.*, 2001). In a more recent study, Botelho *et al.* (2012) examined the effects of commercial formulations of glyphosate and 2,4-D on *E. coli* toxicity. This study concluded that although there was a small decrease in

growth compared to the control, the herbicides may not be toxic to *E. coli* (Botelho *et al.*, 2012). However, the herbicide concentrations used in this study were low (2,4-D – 0.23 ppm and Roundup – 0.09 ppm). The MRLs set by Codex Alimentarius are up to 400 ppm for 2,4-D and 500 ppm for Roundup (Codex Alimentarius, 2014). Hence, organisms are likely to be exposed to higher herbicide concentrations that may cause an effect.

According to the United States Environmental Protection Agency (EPA) dicamba and 2,4-D have been classified as not likely to be carcinogenic to humans (United States Environmental Protection Agency, 2005; United States Environmental Protection Agency, 2006). However, a Canadian study on herbicide exposure and non-Hodgkin's disease in men found significantly increased risk of developing the cancer when exposed to dicamba or 2,4-D (McDuffie *et al.*, 2001). On the other hand, a later study on dicamba exposure and the incidence of cancer in the U.S. over 9 years, found that there was no association between exposure to dicamba and the risk of cancer (Samanic *et al.*, 2006).

Although glyphosate has been classified as non-carcinogenic for humans, it has also been linked to increased risk of non-Hodgkin's lymphoma (Chang *et al.*, 2011). When applied at recommended application rates, glyphosate has also been shown to reduce earthworms in soils and prevent the formation of cocoons or juveniles (Casabe *et al.*, 2007; Correia & Moreira, 2010). Glyphosate has also been shown to affect: the livers of pregnant rats and their foetus (Beuret *et al.*, 2005), muscle and liver cells in dairy cows (Krüger *et al.*, 2013) and the reproductive system in ducks (Oliveira *et al.*, 2007).

Due to the mode of action of glyphosate, there is increasing concern over the effects it may have on microbial communities. Glyphosate has been shown to alter the soil microbial community in fields where glyphosate was applied at the recommended application rates (Kremer & Means, 2009). There was a shift toward the fungal species – *Fusarium* and a subsequent decrease in *Pseudomonas* species in fields planted with glyphosate-tolerant soybeans and maize (Kremer & Means, 2009). Glyphosate was also shown to affect the gut microbiota of chickens by inhibiting beneficial bacteria (Shehata *et al.*, 2013). A commercial

formulation of glyphosate inhibited the growth of microbes that are used in the food industry, at concentrations below the recommended application rates (Clair *et al.*, 2012).

In a study funded by Monsanto, Williams *et al.* conducted a comprehensive in-depth review in 2011 on the development and reproductive safety of glyphosate. By analyzing the findings of various studies, it was concluded that there is "no solid evidence linking glyphosate to adverse development and reproductive effects at environmentally realistic exposure concentrations" (Williams *et al.*, 2011). Despite some valid points in assessing the validity of studies, some of the results were not accepted because commercial formulations of glyphosate were used or the concentrations tested were above the concentrations humans are likely to be exposed. However, other studies that show the adverse effects of glyphosate such as the effect of glyphosate on duck testis used concentrations within the MRLs range and the recommended application rates (Oliveira *et al.*, 2007).

The debate of whether the use of herbicides is safe continues as many maintain that there may be unknown long-term effects on human health and the environment (Green, 2012). To reduce the environmental impact of dicamba, recently Cojocaru *et al.* (2013) synthesized new forms of dicamba that are hydrophobic ionic liquids. These forms of dicamba had increased efficiency and reduced volatiles, this would reduce the amount of dicamba applied on crops (Cojocaru *et al.*, 2013). Use of such herbicides in the future may reduce the effects on non-target organisms.

1.1.6 Risk assessments of herbicides

Although herbicides fulfill the role of protecting yield losses due to unwanted weeds, they pose risks to non-target organisms. Hence, herbicides are subject to risk analysis before they can be approved for commercialisation. Regulatory agencies such as the U.S. EPA evaluate pesticides to ensure that they can be used without unreasonable risks to human health and the environment (United States Environmental Protection Agency, 2006). The risk assessments for human health include assessing the dietary exposure from food and drinking water as well as from occupational and non-occupational exposures (United States Environmental Protection Agency, 2006).

The environmental risk assessments consist of evaluating the environmental fate and transport of herbicides and their toxicity to non-target organisms (United States Environmental Protection Agency, 2006). A risk quotient is calculated by the U.S. EPA, based on estimated environmental and lethal concentrations. When the agency finds that there is potential for adverse effects on non-target organisms, more stringent assessments focusing on the use, toxicity and fate are undertaken. In addition, the toxic effects on non-target terrestrial or aquatic organisms from other studies are also considered (United States Environmental Protection Agency, 2006). However, this process may fail to identify adverse effects of the herbicide on the environment apart from toxicity. For example, a genome-wide transcriptome study of *E. coli* exposed to glyphosate found that genes apart from those involved in the shikimate pathway were also affected (Lu *et al.*, 2013). Treatment with glyphosate caused an up-regulation of genes involved in cell mobility and chemotaxis and at high glyphosate concentrations growth of *E. coli* was inhibited (Lu *et al.*, 2013).

Many of the tests in place for herbicide risk assessment do not include the effects of herbicides in combinations (Soloneski & Larramendy, 2011). Multiple herbicide residues may persist in the environment (Ensminger *et al.*, 2013) which may combine to cause an effect on non-target organisms. Therefore, the effects of multiple herbicides must also be tested as part of the risk assessment process.

Under some circumstances and even in the most aggressive risk assessments it may not be possible for the risk analysts to identify every possible interaction combination (Rajan & Letourneau, 2012). This could lead to hazardous outcomes once the herbicide is used in the environment. For example, in Bt corn, the endotoxins were allowed to be expressed in pollen, which were transported by wind to other plants (Rajan & Letourneau, 2012). The risk assessment did not take into account the effect of the toxic pollen on organisms that feed on the other plants. It was assumed that these insects would not be harmed because they do not feed on corn plants (Rajan & Letourneau, 2012).

Due to multiple pathways through which organisms may be exposed to herbicides, there may be several other risks that are yet to be discovered or considered. One such risk is the

interaction of herbicides with bacteria. Many of the studies on herbicides focus on toxicity to bacteria and few, if any, address effects at sub-inhibitory concentrations. It is vital that the effects on bacteria are also examined as they are important members of the environment contributing towards decomposition, nutrient cycling and energy flow (Botelho *et al.*, 2012). In the environmental risk assessments conducted by the U.S. EPA for dicamba and 2,4-D, the effects on humans, terrestrial and aquatic animals and non-targeted plants were examined. However, the agency failed to consider the impacts of herbicides on bacteria as part of the environment risk assessment (United States Environmental Protection Agency, 2005; United States Environmental Protection Agency, 2006).

Although glyphosate, dicamba and 2,4-D are considered to be environmentally friendly (Behrens *et al.*, 2007; Wright *et al.*, 2010; Sihtmae *et al.*, 2013), a possible pathway for the adverse effects of these herbicides is yet to be considered. The herbicides at sub-lethal concentrations may affect the bacterial antibiotic response. Potential changes in antibiotic response in bacteria may adversely affect human health and should therefore be included in risk assessments.

1.1.7 Future use of herbicides - Next generation genetically modified crops

According to the Agricultural Resource Management Survey conducted in 2012, a number of U.S. farmers reported a decline in the effectiveness of glyphosate on almost 44% of the area planted with soybeans (National Agricultural Statistics Service, 2014). To reduce the selection pressure or delay the emergence of glyphosate-tolerant weeds, it is recommended that farmers not only rely solely on glyphosate but should also use integrated management systems (Nandula *et al.*, 2005). This includes using herbicides with different modes of action in tank mixtures (Nandula *et al.*, 2005).

Other herbicides that have different modes of action currently being used include 2,4-D and dicamba. However, these herbicides are effective in controlling weeds prior to planting the crop seeds, but are not useable just before or after planting (Crespo *et al.*, 2013). There is increased interest in new herbicide-tolerant crops that are tolerant to more than glyphosate (Green,

2014). However, there has been a drought in the development of new herbicides having new modes of action in the last 20 years (Green, 2012; Green, 2014).

To help extend the use of the currently available herbicides, agrochemical companies are promoting 'second-generation' crops that are tolerant to additional herbicides (Mortensen *et al.*, 2012). A number of multiple-herbicide-tolerant crops have been announced and are pending approval, including combinations of glyphosate, glufosinate and either 2,4-D or dicamba tolerant soybeans and cotton (Wright *et al.*, 2010; Green, 2014). Soybeans, corn and tobacco plants tolerant to dicamba have already been developed by introducing a dicamba monooxygenase, an enzyme that degrades dicamba to an inactive form (Behrens *et al.*, 2007; Cao *et al.*, 2011). The expression of this enzyme in plants provides protection from the herbicide, up to 7 times higher than the highest application rates for corn (Cao *et al.*, 2011). Crops that have been made tolerant to 2,4-D include cotton and tobacco plants, which have a plasmid containing a 2,4-D monooxygenase, an enzyme that degrades 2,4-D (Bayley *et al.*, 1992).

If these crops are approved and commercialised, we can expect an increase in 2,4-D and dicamba use. It is predicted that 2,4-D use on corn crops in the U.S. would increase 30-fold by 2019 compared to 2010 (Benbrook, 2012). There are some weed species already resistant to dicamba and 2,4-D (Green, 2014). Hence, the concentration of the herbicides used has to be high enough to prevent the selection of weeds resistant to multiple herbicides (Lagator *et al.*, 2013). Companies that are developing the new herbicide-tolerant crops suggest that dicamba and 2,4-D should be applied on top of the currently used concentrations of glyphosate (Mortensen *et al.*, 2012). Thus, the development of additional herbicide-tolerant crops and possible overuse of dicamba and 2,4-D will increase the amount of herbicides applied (Mortensen *et al.*, 2012). This may further increase the risks to human health and the environment (Mortensen *et al.*, 2012).

1.2 Antibiotics

Antibiotics are chemical compounds with antimicrobial activity that are used globally to treat and prevent bacterial infections in humans as well as animals (Kemper, 2008). Important

antibiotics used in human and animal medicine include ciprofloxacin, kanamycin, ampicillin, chloramphenicol and tetracycline (Kemper, 2008). Many antibiotics are also used at sub-therapeutic doses in the animal industry to promote growth and feed efficiency (Chopra & Roberts, 2001; Cheng *et al.*, 2014) or prophylactically to treat bees (Tian *et al.*, 2012a). Several antibiotics are soluble in water and about 90% can be excreted in animal urine and 75% can be excreted from faeces (Kwon, 2011). As much as 95% of the excreted antibiotics may be un-metabolised and can be transferred to soil (via manure or agricultural fertilizer) or can be released into waste water systems (Kemper, 2008; Milic *et al.*, 2013). A recent study found that as much as 46 mg/kg of tetracycline was found in pig manure (Holzel *et al.*, 2010). Some antibiotics are also released into natural waters or drinking water because waste water treatments may not be efficient in removing the antibiotics (Milic *et al.*, 2013). More than 30 antibiotics have been detected in sewage effluent, surface waters, ground and drinking water (Kwon, 2011) although at low concentrations (ppb range) (Milic *et al.*, 2013).

Despite the importance of antibiotics, their frequent use is causing increased concern regarding adverse effects they may have on the environment (Milic *et al.*, 2013) and human health (Kemper, 2008). The main concern is the spread of bacteria that are tolerant to higher doses of antibiotics which pose risks to human and animal health (Kemper, 2008; Milic *et al.*, 2013). Antibiotics used in animal husbandry may select for tolerant bacteria which may be transferred to humans via direct contact with animals or through the food chain (Kemper, 2008; Heuer *et al.*, 2011). The use of a single antibiotic may also give rise to multidrug tolerant bacteria (Hao *et al.*, 2014).

In 2006, the use of antibiotics as growth promoters was banned in Europe in order to reduce the environmental and health risks (Milic *et al.*, 2013). Antibiotics continue to be used in the U.S. with at least 17 classes of antibiotics approved for growth promotion (Milic *et al.*, 2013). However, in recent years there has been an effort to reduce the use of these antibiotics (Key & McBride, 2014).

Of the antibiotics used in animal feed, more than 50% are identical or similar to the antibiotics used to treat humans (Milic *et al.*, 2013; Key & McBride, 2014). Hence, the development of antibiotic-tolerant bacteria could be a serious threat to public health (Key & McBride, 2014).

1.2.1 Modes of action of antibiotics

1.2.1.1 Chloramphenicol

Chloramphenicol, a bacteriostatic antibiotic, is a specific and potent inhibitor of bacterial protein synthesis (Schwarz *et al.*, 2004). It binds reversibly to the peptidyltransferase center at the 50S ribosomal subunit of the 70S ribosomes (Schwarz *et al.*, 2004). This prevents the proper orientation of tRNA in the peptidyltransferase center and therefore interferes with protein synthesis by preventing peptide bond formation between amino acids (Dunkle *et al.*, 2010).

1.2.1.2 Tetracycline

Tetracycline, discovered in 1953, is a broad spectrum bacteriostatic antibiotic that inhibits bacterial protein synthesis (Chopra & Roberts, 2001; Griffin *et al.*, 2011). It binds to the 30S ribosomal subunit and subsequently inhibits the binding of aminoacyl-tRNA at the acceptor (A) site in the ribosome, ceasing protein synthesis (Brodersen *et al.*, 2000; Griffin *et al.*, 2011).

1.2.1.3 Ciprofloxacin

Ciprofloxacin is part of the quinolone family of antibiotics and exhibits bactericidal activity (Vilfan *et al.*, 2003). It binds to DNA and topoisomerase II (DNA gyrase) or topoisomerase IV (Hawkey, 2003). These enzymes are involved in DNA replication, transcription, repair, strand supercoiling repair and recombination (Hawkey, 2003). DNA gyrase introduces negative supercoils into DNA to relax the stress formed during transcription and replication (Hawkey, 2003). It does this by hydrolysing the phosphodiester backbone of one DNA strand, passing one region through another and ligation (Hawkey, 2003; Mustaev *et al.*, 2014). Formation of the quinolone-DNA-enzyme complex causes a conformational change in the DNA gyrase which breaks DNA but does not re-ligate the broken DNA strands (Hawkey, 2003; Cheng *et al.*, 2013; Mustaev *et al.*, 2014). This inhibits DNA replication because it blocks movement of the

replication fork and causes chromosome fragmentation (Cheng *et al.*, 2013). This triggers the SOS response and at high enough concentrations causes death (Aldred *et al.*, 2014).

1.2.1.4 Ampicillin

Ampicillin is a penicillin β-lactam antibiotic that has bactericidal activity (Kohanski *et al.*, 2010). It covalently binds to penicillin binding proteins within the cell wall (Yao *et al.*, 2012). The bacterial cell wall is made up of peptidoglycan layers which help balance turgor pressure and give mechanical strength (Kohanski *et al.*, 2010). The peptidoglycan layers are made up of peptide crosslinks which are formed by the transpeptidase activity of the penicillin binding proteins (Yao *et al.*, 2012). When ampicillin binds to the penicillin binding proteins, it blocks the formation of crosslinks which is the last stage of peptidoglycan formation (Yao *et al.*, 2012). The inhibition of peptidoglycan synthesis in combination with active degradation of the peptidoglycan layer (by peptidoglycan hydrolases) leads to an excess of autolysin which induces cell lysis (Kohanski *et al.*, 2010).

1.2.1.5 Kanamycin

Kanamycin is an aminoglycoside antibiotic with bactericidal activity (Kohanski *et al.*, 2010). It binds to the A-site of the 30S ribosomal subunit (Kotra *et al.*, 2000). The A-site is the decoding region that enables codon and anticodon recognition (Kotra *et al.*, 2000). The binding of kanamycin to this site interferes with the recognition of tRNA by rRNA (Kotra *et al.*, 2000). This increases the addition of inappropriate amino acids into the elongating peptide strand and leads to protein mistranslation (Kohanski *et al.*, 2010). These mistranslated proteins can be incorporated into the cytoplasmic membrane, compromising the membrane integrity and increasing the uptake of additional kanamycin (Kohanski *et al.*, 2010). When sufficient kanamycin is within the cell, the ribosome is inhibited and induces oxidative stress which leads to cell death (Kohanski *et al.*, 2008; Kohanski *et al.*, 2010).

1.2.2 Antibiotic tolerance

The introduction of antibiotics in medicine gave new hope for the treatment of infectious diseases (Fernandez *et al.*, 2011). However, over the past decades there has been a rapid

increase in antibiotic tolerant strains, reducing the success of antibiotics (van Hoek *et al.*, 2011; Schaeberle & Hack, 2014). The World Health Organization (WHO) considers that the emergence of antibiotic tolerance is one of the biggest threats to human health (Schaeberle & Hack, 2014). With the wide spread use of antibiotics, several bacterial species have developed increased tolerance (Levy & Marshall, 2004). The concentration of ciprofloxacin that was effective in inhibiting the growth of *Pseudomonas aeruginosa* was less than 0.5 μg/ml in 1990 but in 2008 the Minimum Inhibition Concentration (MIC) rose to 32–64 μg/ml (Fernandez *et al.*, 2011). In addition, antibiotics that were once effective against infectious bacteria are now ineffective and can no longer be used for treatment (Levy & Marshall, 2004). For example, penicillin is no longer used to treat *Streptococcus pneumonia* (Fernandez *et al.*, 2011). It has come to a point where there are only a handful of antibiotics that can be used to treat particularly tolerant strains (Levy & Marshall, 2004). For example, carbapenem antibiotics were one of the last resort antibiotics used to treat life threatening infections of drug-tolerant *Enterobacteriaceae* (Schwaber *et al.*, 2011). However, there have been increasing reports of *Enterobacteriaceae* that are tolerant to carbapenem (Schwaber *et al.*, 2011). The treatment of multidrug-tolerant bacteria is not only costly but also puts many patients' lives at risk (Aminov, 2010). It is estimated that about 25,000 patients die each year from infections caused by multiple-tolerant bacteria in the European Union (Aminov, 2010).

Despite the increased effort to discover or synthesize new drugs for treatment, there has been a decrease in the development of new antibiotics (Cheng *et al.*, 2014; Schaeberle & Hack, 2014). In fact, there have been no new classes of antibiotics to treat Gram-negative bacteria, such as *E. coli* in over 40 years (Stanton, 2013). Over the last few years, however, new antibiotics have been launched and two new antibiotics that treat Gram-positive bacteria have been approved for human use in 2011 and 2012 (Aminov, 2010; Butler *et al.*, 2013).

There are three major types of antibiotic tolerance – intrinsic, acquired and adaptive (Fernandez & Hancock, 2012). Intrinsic tolerance is a naturally occurring phenomenon that is present in all bacterial species (Alekshun & Levy, 2007; Cox & Wright, 2013). It includes all inherent properties that are characteristic of a particular species which limit the action of antibiotics (Fernandez & Hancock, 2012). These however, are independent of antibiotic

selection or horizontal gene transfer (Cox & Wright, 2013). An example of intrinsic tolerance is the multi-drug tolerant phenotype of many Gram-negative bacteria (Cox & Wright, 2013). The possession of a semi-permeable outer membrane and expression of efflux pumps restrict the accumulation of the drug which makes the bacteria insensitive to many antibiotics including those that are clinically effective against Gram-positive bacteria (Fernandez & Hancock, 2012; Cox & Wright, 2013).

Acquired tolerance is when susceptible bacteria become tolerant through genetic change (Fernandez & Hancock, 2012). This could be through incorporation of new genetic material, e.g. through horizontal gene transfer, such as plasmids and transposons which may carry resistance genes or through mutations in the existing genes (Alekshun & Levy, 2007; Fernandez & Hancock, 2012). The incorporation of new genetic material generally mediates high-level tolerance (Levy & Marshall, 2004). Although some mutations can cause high-level tolerance, the majority of mutations cause low-level tolerance (Levy & Marshall, 2004). It is believed that the small increases in tolerance can accumulate and lead to high-level tolerance which can be passed onto daughter cells (Fernandez & Hancock, 2012).

Adaptive tolerance is when bacteria gain a temporary increase in tolerance due to changes in gene and/or protein expression that are triggered by particular environments (Fernandez & Hancock, 2012). Environments that cause stress, limit nutrients or that contain sub-lethal concentrations of antibiotics may trigger the response (Fernandez & Hancock, 2012). Unlike intrinsic or acquired tolerance, adaptive tolerance is phenotypic and is not transmitted vertically to the next generation; instead the tolerance is lost when the bacteria are removed from the inducing environment (Fernandez *et al.*, 2011; Fernandez & Hancock, 2012).

The mechanisms used by bacteria to gain antibiotic tolerance are diverse and can be specific or non-specific (Fernandez & Hancock, 2012). These mechanisms include: changes to the bacterial cell wall which restricts the antibiotic from reaching the target site within the cell, the active efflux of an antibiotic from the cell, enzymatic modification or degradation of the antibiotic, modification of the target site, overproduction of the target enzyme and acquisition of

alternative pathways to those inhibited by the antibiotic (van Hoek *et al.*, 2011). For the purposes of this study, active efflux and decreased antibiotic entry will be discussed further.

1.2.2.1 Efflux pumps and porins

One of the modes of tolerance toward many antibiotics, including ampicillin, tetracycline, ciprofloxacin, chloramphenicol and kanamycin, is through efflux pumps (Davies & Davies, 2010) which are widespread in microorganisms (Cox & Wright, 2013). Efflux pumps, first described in 1980, facilitate the transport of antibiotics from the cytoplasm and across the inner and outer membrane of the cell envelope (Alekshun & Levy, 2007; Martinez *et al.*, 2009). The increase in active efflux is of concern because multidrug efflux pumps can cause tolerance to a number of antibiotics (Li & Nikaido, 2009). Chromosomally-encoded efflux pumps in bacteria have been categorised into five main families: the resistance-nodulation-cell division (RND) superfamily, major facilitator (MF) superfamily, small multidrug resistance (SMR) family, ATP-binding cassette super (ABC) family and the multidrug and toxic compound extrusion (MATE) family (Alekshun & Levy, 2007; Li & Nikaido, 2009). Due to efflux pumps being present in bacteria that do not produce antibiotics it is thought that the pumps evolved to avoid toxins in general, not just antibiotics (Cox & Wright, 2013). Efflux pumps can be substrate specific, transporting only one molecule, or can have a broad-spectrum enabling the transport of structurally distinct molecules (Cox & Wright, 2013).

RND family efflux pumps are capable of binding multiple structurally unrelated compounds which enables tolerance to a broad range of antibiotics (Alekshun & Levy, 2007; Li & Nikaido, 2009). They are composed of three distinct elements: a RND transporter which crosses the cell membrane, a membrane-fusion linker protein which sits in the periplasmic space and an outer membrane protein that connects to the outside of the cell (Cox & Wright, 2013). One of the well-studied efflux pumps of the RND family is the AcrAB-TolC pump (Li & Nikaido, 2009). This pump forms a tripartite complex, where AcrB is the transporter, AcrA is the membrane-fusion linker and TolC is the outer membrane protein that joins AcrA to the outside of the cell (Li & Nikaido, 2009). AcrB is able to pump a wide range of antibiotics except aminoglycosides (Lim & Nikaido, 2010). In *E. coli* the AcrAB-TolC pump contributes to the resistance toward

tetracyclines, fluoroquinolones, β-lactams (Cox & Wright, 2013) and chloramphenicols (Okusu *et al.*, 1996; Schwarz *et al.*, 2004).

The AcrAB-TolC efflux pump also exists in *Salmonella typhimurium* and it is highly similar to the efflux pump in *E. coli*. For example, AcrB is 96% identical to the corresponding homolog in *E. coli* at the genomic level (Eaves *et al.*, 2004). A study by Baucheron *et al.* (2004) showed that overproduction of the AcrAB-TolC pump mediates tolerance to quinolones, chloramphenicol and tetracycline in multidrug tolerant *S. typhimurium* (Baucheron *et al.*, 2004). In that study, a strain with mutant *acrB* or *tolC* decreased the MIC for ciprofloxacin from 2 μg/ml to 0.06 μg/ml and for chloramphenicol from 256 μg/ml to 16 μg/ml (Baucheron *et al.*, 2004). However, tolerance towards ampicillin was not affected by the *acrB* and *tolC* mutants (Baucheron *et al.*, 2004). On the other hand, in previous studies, strains that overproduced the AcrAB efflux pump were shown to have increased tolerance to β-lactams with lipophilic side chains such as penicillin (Nikaido *et al.*, 1998). Although both penicillin and ampicillin are only moderately lipophilic, ampicillin is less lipophilic (Glover *et al.*, 1996) which could explain why ampicillin tolerance was unaffected in *acrB* and *tolC* mutants.

S. typhimurium also possesses an AcrD efflux protein which associates with AcrA and TolC to form a tripartite complex (Eaves *et al.*, 2004; Sidhu *et al.*, 2012). This efflux pump is 79% identical to AcrB (Eaves *et al.*, 2004; Piddock, 2006). It was shown to facilitate the transport of aminoglycosides such as kanamycin in *E. coli* (Rosenberg *et al.*, 2000). The active efflux of the antibiotics protects the cell from the antimicrobial effects. For example, when the pump exports tetracycline from the cell, it reduces the intracellular concentrations which protect the ribosome (Chopra & Roberts, 2001). Active efflux of antibiotics alone does not generally cause high-level tolerance, however, a synergistic relationship between active efflux and decreased permeability of the outer membrane can cause high-level tolerance (Cox & Wright, 2013).

The outer membrane of Gram-negative bacteria acts as a barrier to the environment and restricts the entry of toxic chemicals, protecting the cell (Alekshun & Levy, 2007; Ziervogel & Roux, 2013). To allow the entry of nutrients into the cell, bacteria have evolved proteins called porins which function as nonspecific entry and exit points for small hydrophilic molecules

including antibiotics (Fernandez & Hancock, 2012). Porins form β-barrels and are located in the outer membrane (Fernandez & Hancock, 2012). OmpF is one such major porin in *E. coli* and *S.°typhimurium* (Ziervogel & Roux, 2013). A decrease in porin expression is associated with reduced accumulation of antibiotics in the cell which increases tolerance (Nikaido & Pages, 2012; Ziervogel & Roux, 2013). In *E. coli*, loss of this trimeric OmpF porin causes resistance to β-lactams (Fernandez & Hancock, 2012) and in *S. typhimurium* chloramphenicol tolerance was attributed to a lack of OmpF porins (Balasubramaniam *et al.*, 2012).

The production of efflux pumps and porins is controlled by the *mar* (multiple antibiotic resistance) locus which is located on the bacterial chromosome (Alekshun & Levy, 1999). In *E. coli*, MarR is a repressor and negatively controls the expression of the *marRAB* operon (Piddock, 2006). Under non-inducing conditions, MarR binds to the promoter region which is within the *mar* operator and prevents the transcription of the *marRAB* operon (Jaktaji & Heidari, 2013). If MarR is inactivated by chemicals, it allows the expression of the *marRAB* operon (Alekshun & Levy, 1999). However, the level of expression is dependent on several transcription activators such as MarA, Rob and SoxS (Alekshun & Levy, 1999).

Overexpression of MarA and SoxS activates the expression of *acrAB*, causing an increase in the number of efflux pumps (Piddock, 2006). MarA and SoxS also control the expression of porin proteins (Piddock, 2006). The overexpression of the transcriptional activators causes expression of *micF* (Jaktaji & Heidari, 2013). *micF* is an antisense RNA which base pairs to the ompF mRNA forming a duplex and inhibiting translation (Jaktaji & Heidari, 2013). Therefore, expression of the transcriptional activators enhances the efflux of antibiotics that are substrates of the AcrAB-TolC efflux pump and decrease the entry of antibiotics due to reduced production of OmpF porins (Piddock, 2006; Jaktaji & Heidari, 2013). This increases antibiotic tolerance (Jaktaji & Heidari, 2013). Due to the significant sequence similarities between the *E. coli* operons and pumps and those of *S. typhimurium*, it is thought that the regulation of influx and efflux in *S. typhimurium* is similar to that of *E. coli* (Piddock, 2006; Zheng *et al.*, 2009). For example, elevated levels of *ramA* transcription (a transcriptional activator), has been shown to increase the expression of *acrAB* in multidrug tolerant *S. typhimurium* (Zheng *et al.*, 2009).

1.3 Effect of salicylic acid on antibiotic tolerance

Salicylic acid and its derivatives have been reported to induce an antibiotic response in multiple bacterial species to antibiotics that are structurally unrelated and have different targets and mode of actions (Foulds *et al.*, 1989; Berlanga & Vinas, 2000; Price *et al.*, 2000; Koney & Morse, 2009). Salicylic acid, a weak acid, is a naturally produced phenolic molecule that is widely used by plants as a defense compound (Wu *et al.*, 2012). It is also used by humans in cosmetics and medicine, e.g. derivatised as an active component of aspirin (Hartog *et al.*, 2010; Tian *et al.*, 2012b). Its structure is made up of a benzene ring and has a carboxylic acid component (Fig. 1.1). Interestingly, the chemical structure of dicamba and 2,4-D have a salicylic acid backbone, while glyphosate is not structurally similar to salicylic acid (Fig. 1.1).

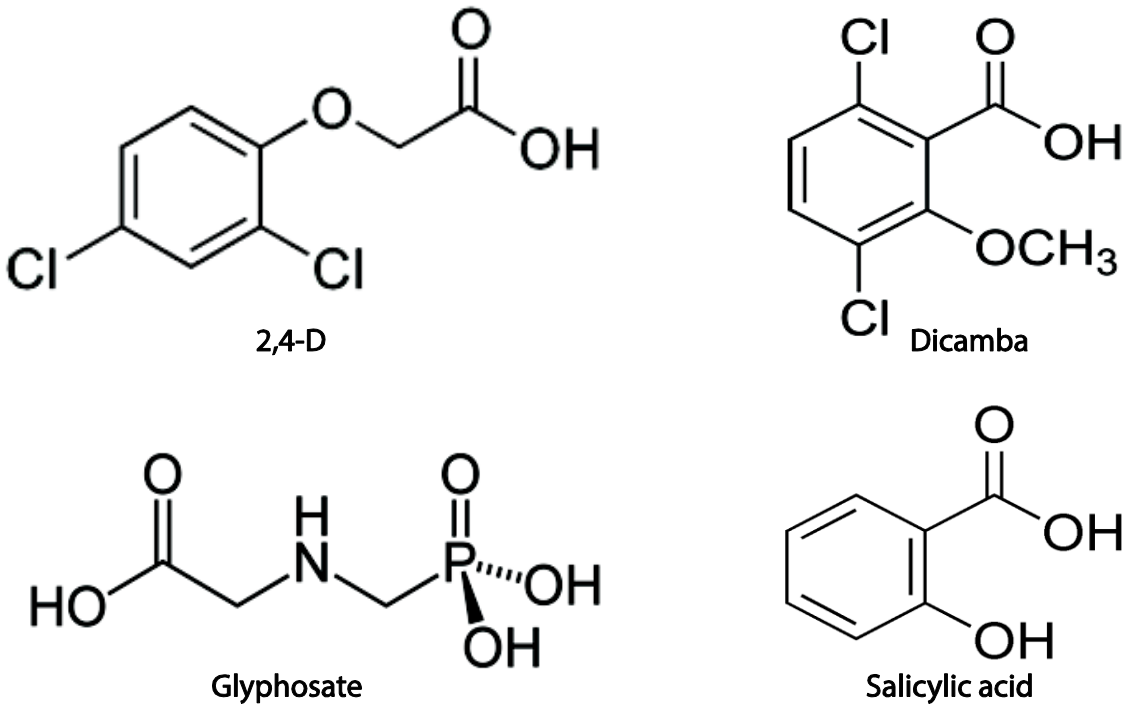

Figure 1.1. Structures of 2,4-D, glyphosate, dicamba and salicylic acid.

In 1985, Judah L. Rosner first reported a salicylic acid induced antibiotic tolerance in *E. coli* towards chloramphenicol, ampicillin, nalidixic acid and tetracycline (Rosner, 1985). Samples of *E. coli* were added to top agar and then plated onto media containing an antibiotic and salicylic

acid (Rosner, 1985). Following incubation, the number of colonies that appeared were converted to efficiency of plating (EOP), which is calculated by dividing the number of colonies appearing on test plates by the number of colonies appearing on control plates (lacking antibiotic and salicylic acid) (Rosner, 1985). At antibiotic concentrations that inhibited bacterial growth, the addition of salicylic acid dramatically increased the EOP. For chloramphenicol the EOP increased from ≤ 0.074 to 0.66-1.05, depending on the strain. When colonies were transferred to a medium without salicylic acid, they showed normal sensitivity to chloramphenicol (Rosner, 1985). Hence, the tolerance induced by salicylic acid was phenotypic (Rosner, 1985). Furthermore, salicylic acid was able to induce multiple drug resistance by increasing the EOP by 10^6-fold or higher (Rosner, 1985).

The effect of salicylic acid has been linked to increased production of efflux pumps and decreased permeability of the outer membrane. In *E. coli*, salicylic acid reduces the repressor activity of MarR by binding to it and preventing it from binding to the *marO* operator region of the *mar* operon (Alekshun & Levy, 1999; Price *et al.*, 2000). This leads to increased production of MarA and subsequent increase in AcrAB efflux pump production and a concomitant reduction in the outer membrane porin OmpF (Berlanga & Vinas, 2000; Price *et al.*, 2000; Li & Nikaido, 2009). Salicylic acid highly and reversibly inhibited the synthesis of the OmpF porin in *E. coli* in a dose-dependent manner (Sawai *et al.*, 1987). The synthesis of the OmpC porin was also weakly inhibited by salicylic acid (Sawai *et al.*, 1987).

Foulds *et al.* (1989) studied the permeation of cephalosporins (a class of β-lactam antibiotics) through the outer membrane of *E. coli* that were grown in salicylic acid. This was done by measuring the rate of β-lactam hydrolysis caused by β-lactamase that was present in the periplasmic space. Results showed a 3 to 5-fold decrease in permeation of the outer membrane compared to cells that were not exposed to salicylic acid (Foulds *et al.*, 1989). Similar to the results gathered by Sawai *et al.* (1987), the effect on permeability was reversible. When cells were transferred to salicylic acid-free medium, permeability returned to normal levels within 3 hours, allowing for the synthesis of OmpF (Foulds *et al.*, 1989). Salicylic acid also inhibited the synthesis of the OprD efflux pump in *P. aeruginosa* which increased carbapenem tolerance (Sumita & Fukasawa, 1993). As previously mentioned, the OmpF porin is an effective pathway

for antibiotics such as chloramphenicol, tetracycline and β-lactams to pass through the outer membrane. Hence, a decrease in OmpF in conjunction with the increase in efflux pumps, reduces the accumulation of antibiotics within the cell allowing the cell to grow at higher antibiotic concentrations (Sawai *et al.*, 1987).

Authors have suggested that salicylic acid may also increase antibiotic resistance through a *mar*-independent pathway in *E. coli* (Cohen *et al.*, 1993; Berlanga & Vinas, 2000). A study that determined the effect of salicylic acid in a *mar*-inactivated strain of *E. coli,* found that salicylic acid was still able to increase tolerance (Cohen *et al.*, 1993). The amount of OmpF in *mar* deleted strains was also reduced when salicylic acid was added to the growth media (Cohen *et al.*, 1993). However, the level of antibiotic tolerance that was induced was lower in the *mar* deleted strains than the wild type strains (Cohen *et al.*, 1993). Hence, the effect of salicylic acid is mainly mediated by the *mar* operon but may also be mediated by an unidentified *mar*-independent pathway (Cohen *et al.*, 1993).

S. typhimurium also exhibited increased chloramphenicol and ciprofloxacin tolerance when salicylic acid was present in the growth medium (Price *et al.*, 2000; Randall & Woodward, 2001; Hartog *et al.*, 2010). Exposure to salicylic acid increased the expression of *marA*, *marRAB, acrAB* and *micF* by 2-4, 30.7, 3.6 and 9.6-fold, respectively (Randall & Woodward, 2001; Hartog *et al.*, 2010). The deletion of the *marA* gene increased ciprofloxacin susceptibility even in the presence of salicylic acid (Hartog *et al.*, 2010). In addition, inhibition of the AcrAB efflux pump by Phe-Arg-β-napthylamide (PAβN) diminished the antibiotic tolerance induced by salicylic acid (Hartog *et al.*, 2010).

Salicylic acid has also been shown to affect antibiotic tolerance in other Gram-negative and Gram-positive bacteria. In *Serratia marcescens*, the presence of salicylic acid reduced the bactericidal activity of nalidixic acid and ciprofloxacin (Berlanga & Vinas, 2000). Similar to the effect in *E. coli*, the induced tolerance reverted to the original susceptibility patterns when the bacterial cells were sub-cultured in the absence of salicylic acid (Berlanga & Vinas, 2000). Salicylic acid also repressed porins in *S. marcescens*, significantly decreasing the uptake of ciprofloxacin (Berlanga & Vinas, 2000). In *Staphylococcus aureus*, salicylic acid increased

ciprofloxacin tolerance (Gustafson *et al.*, 1999). Salicylic acid was shown to down-regulate two genes that repress multidrug efflux pumps in *S. aureus*, allowing the up-regulation of efflux pumps and enabling *S. aureus* to resist antibiotic stress (Riordan *et al.*, 2007).

Salicylic acid does not induce antibiotic tolerance to all antibiotics; instead it can increase the antimicrobial activity of some, particularly aminoglycosides (Price *et al.*, 2000). *E. coli* exposed to salicylic acid did not show tolerance to kanamycin (Rosner, 1985); rather they became more susceptible, reducing the EOP by more than 10^4-fold (Aumercier *et al.*, 1990). Although it is not clear which mechanism causes this effect, it is thought that when salicylic acid enters the cell it increases the membrane potential which facilitates the entry of aminoglycosides into the cell (Aumercier *et al.*, 1990; Price *et al.*, 2000). However, it may also be due to decreased expression of the AcrD efflux pump which enables the efflux of aminoglycosides through the outer membrane.

1.4 Objectives of this study

The main objective of this study was to investigate potential effects of commercial formulations of dicamba, glyphosate and 2,4-D on bacteria with adverse consequences for medicine, a particular focus being on antibiotic tolerance phenotypes. *E. coli* and *S. typhimurium* were chosen as model organisms with my responsibility being studies in the latter. *S. typhimurium* is a pathogen that is known to cause a number of human diseases, including gastroenteritis, bacteremia and typhoid fever (Nikaido *et al.*, 2008). Since the 1990s, there has been an increase in the number of multidrug-tolerant *S. typhimurium* in various countries and has been found in both humans and animals, causing concern for public health (Nikaido *et al.*, 2008).

The aims of this study were:

- To determine the herbicide concentrations that are toxic to *S. typhimurium;*
- To investigate if the herbicides induced an antibiotic response towards chloramphenicol, tetracycline, ciprofloxacin, ampicillin and kanamycin;
- To determine the minimum herbicide concentrations necessary to induce an antibiotic response; and

- To determine if the induced antibiotic tolerance is maintained in the absence of the herbicide.

Chapter Two

2. The Toxic Effect of Herbicides on Microbes and their Ability to Induce an Antibiotic Response

2.1 Introduction

The rapid adoption of genetically modified plants by some countries since 1996 has led to a dramatic increase in the use of herbicides, particularly glyphosate-based herbicides such as trade named Roundup (Benbrook, 2012). The increase in glyphosate use and subsequent reduction of other herbicides (Nandula *et al.*, 2005) has caused a monoculture of herbicide. This has increased the selection pressure favouring glyphosate-tolerant weeds (Benbrook, 2012). There are 215 reported glyphosate-tolerant weed populations according to the International Survey of Herbicide Resistant Weeds (Heap, 2014b).

In response to glyphosate-tolerant weeds, crop plants tolerant to other herbicides such as dicamba are in the process of development (Service, 2007; Cao *et al.*, 2011). Furthermore, crops tolerant to dual herbicides – 2,4-D and glyphosate (Li *et al.*, 2013), dicamba and glyphosate (Dorey, 2013) are also being engineered. With the development of such herbicide tolerant crops, an increase in dicamba, 2,4-D and glyphosate use is anticipated (Benbrook, 2012).

There is some knowledge about the toxicity of these herbicides. Most of these studies focus on the effects herbicides have on mammalian cell lines (Bukowska, 2006; Gonzalez *et al.*, 2006) and aquatic organisms (Relyea, 2005). Studies relevant to soil microbial communities or industrial microorganisms are limited to toxicity (Busse *et al.*, 2001; Clair *et al.*, 2012), and not other relevant effects. Before dicamba and 2,4-D tolerant crops are cultivated it is vital to have a better understanding of the effects these herbicides may have on the microbial community and wider environment. The effect of commercial formulations of the three herbicides: Kamba, 2,4-D and Roundup on bacterial toxicity was tested in this study. Herbicide toxicity was

determined by establishing Minimum Inhibitory Concentrations (MIC) of the three herbicides for *Salmonella enterica* serovar *Typhimurium*, in solid and liquid media. *S. typhimurium* was chosen as a model organism because it is a potential animal and human pathogen and because it has different physical and animal vectors through which it can enter the food and feed chain.

Herbicides are chosen by their effects on plants. However, biocides and other chemicals introduced to an environment can have unintended side effects on non-target organisms. For example, Roundup was found to reduce species richness in aquatic communities (Relyea, 2005). As mentioned previously, unintended effects tend to be described for toxicity only. The effect of herbicides at sub-lethal concentrations on microbes remains unclear. Potential side effects of Kamba, 2,4-D and Roundup on *S. typhimurium* were tested, focusing on changes of another type. The change we monitored was on subsequent changes in response to antibiotics.

Previous studies have shown that salicylic acid can cause tetracycline, chloramphenicol and ampicillin tolerance in *Escherichia coli* (Rosner, 1985; Cohen *et al.*, 1993). This similarity in structure of dicamba and 2,4-D to salicylic acid prompted the investigation of whether commercial formulations of these herbicides are able to cause antibiotic tolerance phenotypes in *S. typhimurium*. Because glyphosate does not share structural similarity with salicylic acid it was initially included as a control.

Similar to how the original studies with salicylic acid were done, *S. typhimurium* was cultured in the presence of an antibiotic and an herbicide. The number of bacterial cells that grew in the presence of the herbicide was compared to the growth in the absence of herbicide. The results were converted to an expression called Efficiency Of Plating (EOP) (Rosner, 1985; Cohen *et al.*, 1993). The potential changes in antibiotic tolerance were tested using antibiotics of different classes: tetracycline, kanamycin, ciprofloxacin, chloramphenicol and ampicillin. The ability of salicylic acid to cause antibiotic tolerance in *S. typhimurium* was confirmed in this study.

2.2 Materials and methods

2.2.1 Bacterial strain, culture conditions and chemicals

Salmonella enteric serovar *Typhimurium* LT2 strain SL3770 (*rfa*$^{+}$) was used for this study (MacLachlan & Sanderson, 1985). Master stocks were stored at -80^{0}C in glycerol storage medium. The culture was maintained on Luria Broth Agar and grown in Luria Broth (LB) before experiments. All cultures were incubated at 37^{0}C. Liquid cultures were grown in a Gyrotory water bath shaker at 215 revolutions per minute. Luria broth Base (Lennox-L-Broth Base) was purchased from Invitrogen and agar (Bacteriological Agar No.1) was purchased from Oxoid.

The antibiotics used in this study were ampicillin (Stock concentration - 100 mg/ml), tetracycline (5 mg/ml), kanamycin (40 mg/ml), ciprofloxacin (10 mg/ml) and chloramphenicol (20 mg/ml). The stock concentrations were stored at -20^{0}C. A 1M solution of salicylic acid (stored at room temperature) was made by dissolving salicylic acid powder in ethanol and the pH neutralized to 7. Salicylic acid, chloramphenicol and tetracycline hydrochloride were purchased from Sigma-Aldrich. Ampicillin sodium salt was purchased from AppliChem, ciprofloxacin hydrochloride from Pentex and kanamycin sulfate from Life Technologies.

The three commercially available herbicides used in this study were Kamba 500, 2,4-D (2,4-Dichlorophenoxyacetic acid) and Roundup. Kamba 500 was manufactured by Nufarm with the active ingredient dicamba as a dimethylamine salt (500 g/L). Roundup weedkiller concentrate had glyphosate (360 g/L) in the form of an isopropylamine salt as the active ingredient. 2,4-D Amine 800 WSG, purchased from Agpro NZ limited, had the active ingredient 2,4-D (800 g/kg) as a dimethylamine salt. The 2,4-D powder was dissolved in sterile water to make a solution of 0.552 Ibs/gal ae (acid equivalent). The acid equivalent is defined as the theoretical yield of the parent acid from the herbicide active ingredient which has been formulated as a salt. For all experiments Kamba 500 was filtered through a 0.2 µm Supor membrane low protein binding non-pyrogenic filter and all herbicides were stored at room temperature.

2.2.2 Determining minimum inhibitory concentrations

2.2.2.1 Solid media

MICs are the lowest concentration of an antibacterial substance that inhibits bacterial growth following overnight incubation (Wiegand *et al.*, 2008). MICs were determined for the five antibiotics and three herbicides which were incorporated into solid media. *S. typhimurium* culture was grown in liquid LB until it reached an optical density (OD_{600}) of 1, measured using a spectroflurometer (Novaspec III-Abs 600 nm). The culture was serially diluted 10-fold in LB and the various dilutions were applied in 10 µl droplets ('spot plating') to plates with increasing concentrations of antibiotic or herbicide. The plates were freshly made and dried for at least 40 minutes in a fume hood. The chosen antibiotic and herbicide concentrations ranged from dilutions that allow bacteria to grow to dilutions that inhibit growth. The final culture dilutions plated (10^{-5} to 10^{-8}) were in duplicate and the plates were incubated at 37^0C for 16–20 hours. The lowest concentration of antibiotic or herbicide that inhibited visible bacterial growth after overnight incubation was taken as the MIC. Bacterial colonies that grew on a plate without any antibiotic or herbicide was used as the control.

The Clinical and Laboratory Standards Institute has set guidelines for the determination of MICs of antimicrobials (Clinical and Laboratory Standards Institute, 2006). For the agar dilution method, the culture inoculums can be prepared by directly inoculating a broth suspension with a colony from an 18-24 hour agar plate. The turbidity is then adjusted to achieve a turbidity equivalent to 0.5 McFarland turbidity standard. This is done so that 1 to 2 x 10^8 cfu/ml is achieved in the suspension. Agar plates are then prepared with various dilutions of the antimicrobial. A drug-free plate is also included as a control. The dilutions are then diluted in order to attain a final concentration of 10^4 cfu/spot. An aliquot of the inoculums is then applied to the agar and once the spots have been absorbed into the agar, the plates are incubated for 16-20 hours at 35^0C to 37^0C. Following incubation, the MIC is recorded as the lowest concentration of antimicrobial agent that completely inhibits growth.

The methods used here are similar to the methods outlined by the Clinical and Laboratory Standards Institute but with a few variations. The turbidity of the culture was not adjusted using the McFarland turbidity standard. Instead the culture was grown to an optical density of 1 at which a minimum of 1 x 10^8 cfu/ml is normally achieved. The inoculum was diluted to various concentrations, four of which were spot plated. The number of colonies that grew ranged from a lawn to a single colony.

2.2.2.2 Liquid media

MICs were also determined using the macro dilution broth medium method. *S. typhimurium* was grown in liquid LB at 37^0C until the culture reached an optical density (OD_{600}) of 1. Different concentrations of antibiotics and herbicides were diluted in LB and pipetted into a 24 well plate. The wells contained antibiotic or herbicide in progressively increasing concentrations, the lower allowing bacterial growth and the higher inhibiting growth. To each condition, 20 µl of the culture was added to give a total reaction volume of 2 ml per well. Controls included LB as the blank and LB plus the bacterial culture as a positive control. Optical density was measured before incubation using a spectroflurometer (NovaspecIII-Abs 600 nm) (1ml). Cultures were then incubated for 16–20 hours at 37^0C in a shaking incubator at 215 rpm. After incubation, growth was measured as a function of optical density at 600 nm on a Novaspec III spectrophotometer. The optical density of each treatment was compared against the no treatment control. The minimum inhibition concentration was defined as the lowest concentration of antibiotic or herbicide that inhibited bacterial growth (difference in optical density before and after incubation, less than 0.1).

Guidelines for broth dilution methods have also been set by The Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2006). There were a few differences between the method used here and the set guidelines. The total volume of the broth in each well was 2 ml instead of 1 ml and the culture was not diluted before inoculating the broth. According to the guidelines, the MIC is the lowest concentration that completely inhibits growth as detected by the unaided eye. Here, the optical density of the culture was

determined before and after incubation. The lowest concentration with an optical difference of 0.1 or less was taken as the MIC.

2.2.3 Determining the effect of herbicides on antibiotic response

2.2.3.1 Spot plates

An assay was performed to determine if herbicides induce an antibiotic response. The three herbicides Kamba (1827 ppm ae), 2,4-D (1940 ppm ae) and Roundup (1243 ppm ae) were tested at sub-lethal concentrations. In addition, salicylic acid (346 ppm) was also tested. Agar plates were made with each of the herbicides and salicylic acid at the given concentrations plus each of the antibiotics ranging from just below MIC and above MIC (previously determined). Controls included plates with just the antibiotic, herbicide or salicylic acid and plates with no chemical treatment.

A *S. typhimurium* culture was grown in LB at 37^0C until the culture reached an optical density (OD_{600}) of 1. The culture was serially diluted 10-fold in LB and 10 µl of each dilution was then spot plated (final dilutions 10^{-2} to 10^{-9}) on the prepared plates. The spots of culture were allowed to dry before incubation at 37^0C for 16-24 hours. The number of colonies (colony forming units – cfu/ml) that grew following incubation were counted (on dilutions with countable numbers) and the EOP, as previously described (Rosner, 1985), was calculated for each condition according to the formula below.

EOP = (Treatment cfu/ml) / (No treatment control cfu/ml)

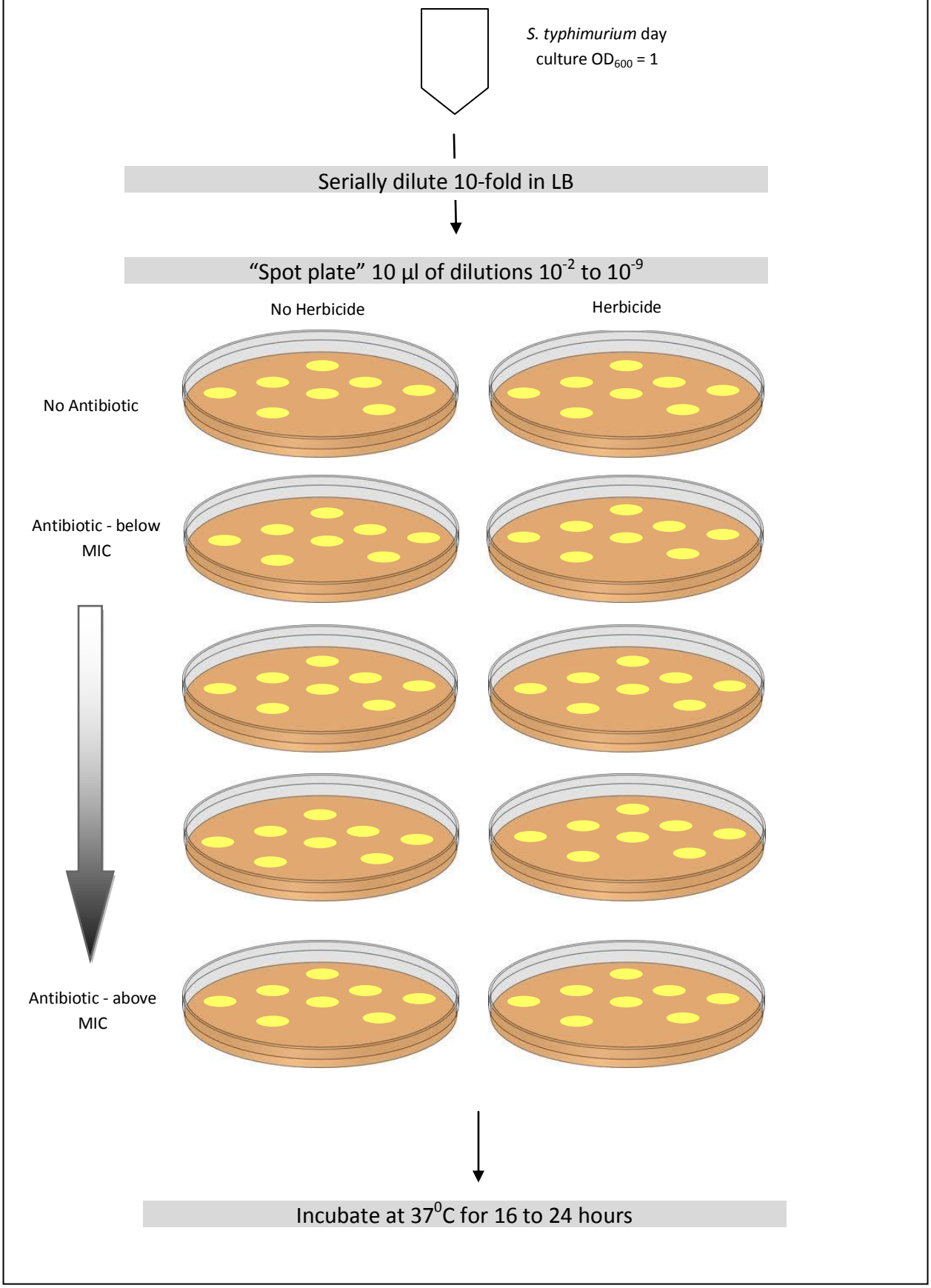

Figure 2.1. Schematic diagram of the assay conducted to test the effect of herbicides on antibiotic response.

2.2.3.2 Broth dilution method

The effect of herbicides in changing antibiotic MIC in *S. typhimurium* was determined using the broth dilution method. *S. typhimurium* culture was grown in LB to an optical density (OD_{600}) of 1 and 10µl was incubated with varying concentrations (low to high) of antibiotics with each of the three herbicides (Kamba 1827 ppm ae, 2,4-D 1940 ppm ae and Roundup 1243 ppm ae) and salicylic acid (346 ppm) in 24 well plates. Controls included: no herbicide treatment, no antibiotic treatment, no chemical treatment as a positive control, and an LB treatment without culture as a blank. Cultures were incubated for 16-20 hours at 37^0C in a shaking incubator and then examined for bacterial growth visually.

2.2.4 Statistical analysis

Statistical power is the probability of rejecting the null hypothesis when it is false and therefore should be rejected (Roush & Tozer, 2004). The free statistical program R (http://www.r-project.org/) was used to analyze data gathered in this study for statistical power. To analyze the data gathered from the assay which determined the effect of herbicides on antibiotic response, the statistical difference between treatments was tested with the null hypothesis. A null hypothesis is defined as 'nothing happening' meaning that there is no difference between two treatments (Crawley, 2005). The salicylic acid treatment and herbicide treatment were compared to the antibiotic-only treatment at each antibiotic concentration. A linear model was created and the average EOP of each replicate was logged and each chemical treatment was compared as a factor of antibiotic concentration. The Bonferroni correction method was then used to reduce the chances of obtaining a false-positive result, correcting the p-values. The p-value is a measure of reliability of the null hypothesis (Crawley, 2005). A low p-value means that the effect seen is unlikely to have occurred by chance and would therefore be statistically significant (Crawley, 2005). The p-value threshold was set at < 0.01, meaning that the likelihood of the observed result occurred by chance alone is less than 1%. Therefore, treatments with p-values below 0.01 were taken as statistically significant.

2.3 Results

2.3.1 Minimum inhibitory concentrations of antibiotics

The lowest concentration of antibiotic that causes antimicrobial activity towards *S. typhimurium* was determined for five antibiotics using spot plating and broth dilution methods. Bacteria were grown under varying concentrations of each antibiotic and bacterial growth for each condition was determined after incubation (16–20 hours). Table 2.1 shows the MICs for each antibiotic determined using culturing on solid media and Table 2.2 shows the MICs determined using culturing in liquid media. The MICs vary slightly between the two methods used, highlighting the sensitivity and variability of MICs in different experimental conditions (Wiegand *et al.*, 2008). MICs may have also been affected by the storage conditions, as the antibiotics were not dehydrated which may have altered the weight. Hence, there is a need to determine MICs in each laboratory. The antibiotic MICs determined here dictated the concentration of antibiotics that were to be used in subsequent experiments.

Table 2.1. Minimum inhibitory concentrations of antibiotics measured on solid media

Antibiotic	MIC (μg/ml)
Ampicillin	2.0 ± 0.0
Tetracycline	1.7 ± 0.1
Kanamycin	8.0 ± 0.0
Ciprofloxacin	0.05 ± 0.00
Chloramphenicol	5.0 ± 0.0

MICs ± SEM (n=3) were measured on plates by spot plating 10μl of *S. typhimurium* culture. Following 16–20 hours of incubation, at 37^0C, appearance of single colonies was taken as bacterial growth.

Table 2.2. Minimum inhibitory concentrations of antibiotics measured in liquid media

Antibiotic	MIC (µg/ml)
Ampicillin	5.0 ± 0.0
Tetracycline	2.3 ± 0.0
Kanamycin	18.0 ± 2.9
Ciprofloxacin	1.0 ± 0.0
Chloramphenicol	4.0 ± 0.0

MICs ± SEM (n=3) were measured in liquid LB media for *S. typhimurium*. Following 16–20 hours of incubation at 37^0C, the OD_{600} of the cultures were measured. Antibiotic concentrations that inhibited growth (difference in OD_{600}, before and after incubation, less than 0.1) were taken as the MIC.

2.3.2 Herbicide toxicity

The toxicity of Kamba, 2,4-D and Roundup toward *S. typhimurium* was determined by finding the minimum concentration of herbicide that inhibited growth on plates and in liquid media. Appearance of individual colonies on plates was scored as positive for growth. In liquid broth growth was measured by OD_{600}. All three of the tested herbicides were toxic to *S. typhimurium* in both solid (Table 2.3) and liquid media (Table 2.4). The reported MICs are given as parts per million of the acid equivalent (ppm ae). The MICs vary between the two methods used and Kamba appears to be the least toxic herbicide of the three. The herbicide concentrations that were toxic were all above the Maximum Residue Limits (MRLs) for food and feed set by Codex Alimentarius (Table 2.5) suggesting that herbicide residues in food products and animal feed may not affect microbial growth. The herbicide MICs were also above the manufacturer recommended application rates (Table 2.6). Therefore it can be anticipated that exposed bacteria remain viable through the food and feed chain.

Table 2.3. Minimum inhibitory concentrations of herbicides determined on solid media

Herbicide	MIC (ppm ae)
Kamba	14485 ± 1690
2,4-D	5780 ± 304
Roundup	6190 ± 442

MICs ± SEM (n=3) were measured on plates by spot plating 10µl of *S. typhimurium* culture. Following 16–20 hours of incubation, at 37^0C, appearance of single colonies was taken as bacterial growth.

Table 2.4. Minimum inhibitory concentrations of herbicides determined in liquid media

Herbicide	MIC (ppm ae)
Kamba	15737 ± 409
2,4-D	5348 ± 61
Roundup	4897 ± 408

MICs ± SEM (n=3) were measured in liquid LB media for *S. typhimurium*. Following 16-20 hours of incubation at 37^0C, the OD_{600} of the cultures were measured. Herbicide concentrations that inhibited growth (difference in OD_{600}, before and after incubation, less than 0.1) were taken as the MIC.

Table 2.5. Herbicide maximum residue limits

Herbicide	Codex Alimentarius MRL range (ppm ae)
Dicamba	0.01 - 50
2,4-D	0.01 - 400
Glyphosate	0.05 - 500

Maximum residue levels for herbicides set by Codex Alimentarius – International Food Standards. Available at http://www.codexalimentarius.net/pestres/data/pesticides/index.html?lang=en

Table 2.6. Recommended herbicide application rates

Herbicide	Recommended application rate (ppm ae)
Kamba	331 – 2483
2,4-D[2]	3310
Roundup	2653

Herbicide application rates as recommended by manufacturer.

[2.] Concentration used for Knapsack

2.3.3 Kamba-induced antibiotic response

The effect of Kamba in causing an antibiotic response towards five antibiotics in *S. typhimurium* was examined. *S. typhimurium* was grown on plates with Kamba (1827 ppm ae) supplemented with varying concentrations of each of the antibiotics. The concentration of Kamba used in this assay was below the herbicide MIC (Table 2.3) and was above the concentration of salicylic acid (346 ppm) that had an effect on chloramphenicol tolerance (Rosner, 1985). The titres for each condition were converted to EOP.

S. typhimurium that were grown in the presence of Kamba had higher MICs for chloramphenicol, tetracycline, ampicillin and ciprofloxacin. The same trend was observed when bacteria were exposed to salicylic acid, as reported elsewhere (Rosner, 1985; Cohen *et al.*, 1993; Gustafson *et al.*, 1999). The EOP detection limit was 0.001, as spots of cultures from 10^2 to 10^5 dilutions had an opaque film with no obvious colonies. Therefore, concentrations of antibiotic that reduced the EOP to 0.001 or less were taken as the MIC. A summary of the antibiotic MICs under Kamba and salicylic acid treatment are shown in Table 2.7.

Table 2.7. Minimum inhibitory concentrations of antibiotics in the presence or absence of chemical treatment

A

Antibiotic	MIC (µg/ml)		
	(-) Kamba	(+) Kamba	Ratio (+ Kamba/- Kamba)
Chloramphenicol	3.44 ± 0.18	11.0 ± 0.0	3.20
Tetracycline	0.78 ± 0.07	3.0 ± 0.0	3.85
Ampicillin	3.50 ± 0.26	5.67 ± 0.88	1.62
Ciprofloxacin	0.03 ± 0.00	0.08 ± 0.00	2.67
Kanamycin	6.89 ± 0.35	1.2 ± 0.0	0.17

B

Antibiotic	MIC (µg/ml)		
	(-) Salicylic acid	(+) Salicylic acid	Ratio (+ Salicylic acid/- Salicylic acid)
Chloramphenicol	3.44 ± 0.18	8.33 ± 0.29	2.42
Tetracycline	0.78 ± 0.07	2.53 ± 0.20	3.24
Ampicillin	3.50 ± 0.26	7.67 ± 0.44	2.19
Ciprofloxacin	0.03 ± 0.00	0.07 ± 0.00	2.33
Kanamycin	6.89 ± 0.35	3.64 ± 0.14	0.53

MICs ± SEM of antibiotics with or without Kamba (A) (1827 ppm ae) and salicylic acid (B) (346 ppm). *S. typhimurium* cultures were grown in plates (spot plates) and incubated at 37^0C for 16-24 hours.

S. typhimurium grown in chloramphenicol supplemented with Kamba or salicylic acid showed tolerance to higher chloramphenicol concentrations (Fig. 2.2). The presence of Kamba increased the MIC of chloramphenicol 3.2-fold (Table 2.7.A). *S. typimurium* grown in salicylic acid showed a 2.42-fold increase in chloramphenicol MIC (Table 2.7. B).

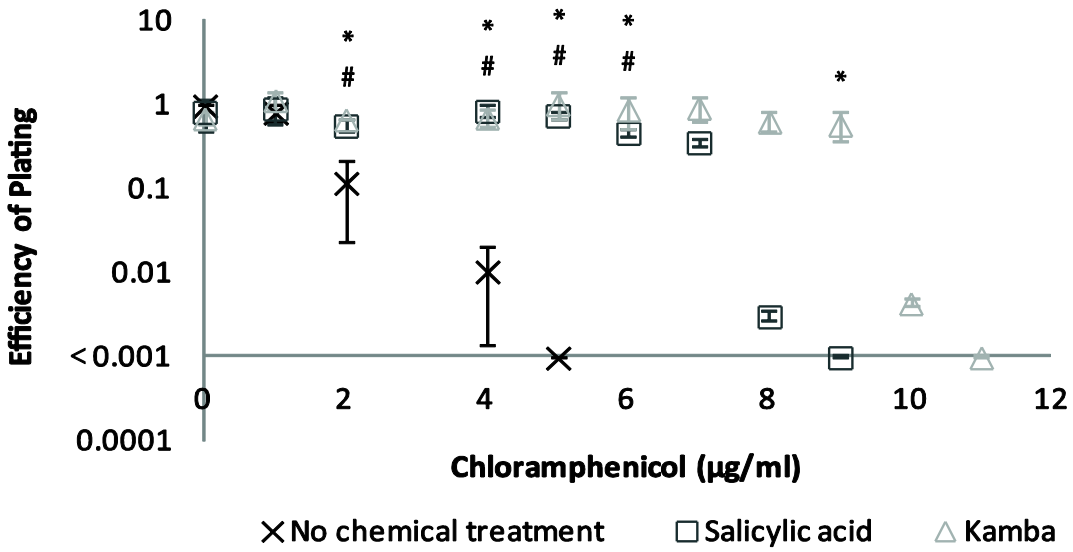

Figure 2.2. Kamba induces chloramphenicol tolerance in *S. typhimurium*.
Bacteria were grown on plates with varying chloramphenicol concentrations with no chemical treatment and on plates with chloramphenicol supplemented with salicylic acid (346 ppm) or Kamba (1827 ppm ae). Results are expressed in EOP ± SEM (n=3). EOPs of the Kamba condition with * are significantly different from the EOPs of the no chemical treatment (p-value < 0.01). EOPs of the salicylic acid condition with # are significantly different to the EOPs of the no chemical condition (p-value < 0.01).

The presence of Kamba and salicylic acid in the media allowed growth of *S. typhimurium* at tetracycline concentrations that would normally inhibit growth (Fig. 2.3). Bacterial cells grown in tetracycline had an MIC of 0.78 µg/ml, however when salicylic acid or Kamba was present, the MIC increased to 2.53 µg/ml and 3.0 µg/ml, respectively (Table 2.7). Similarly, Kamba and salicylic acid increased ciprofloxacin tolerance (Fig. 2.4) by 2.33 and 2.67-fold, respectively (Table 2.7). *S. typhimurium* grown in the presence of Kamba and salicylic acid also showed tolerance to higher ampicillin concentrations (Fig. 2.5). Kamba increased the MIC of ampicillin by 1.62-fold, while salicylic acid increased it by 2.19-fold (Table 2.7).

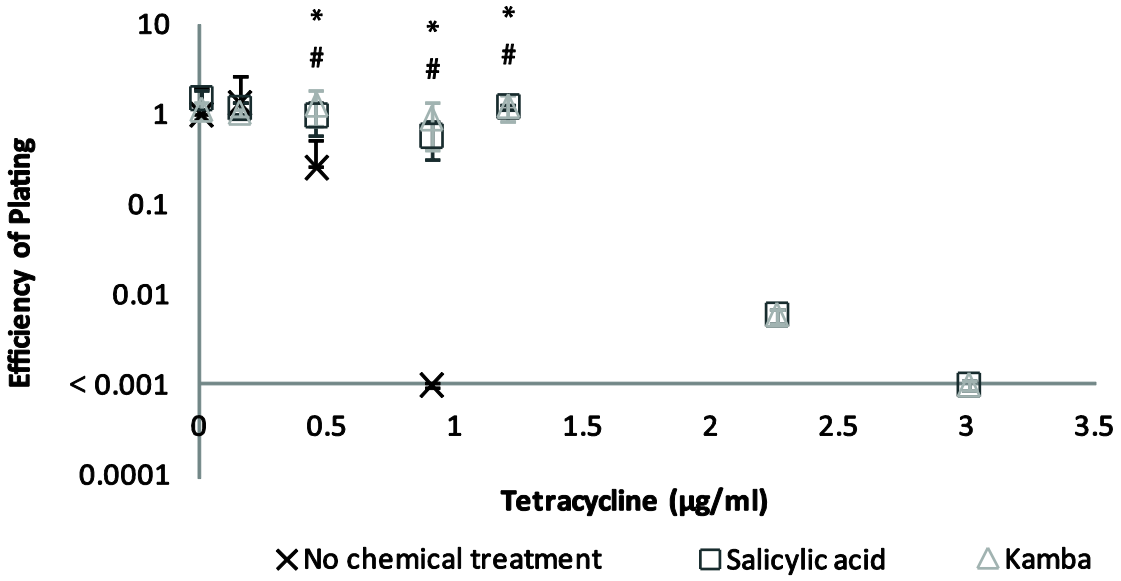

Figure 2.3. Kamba induces tetracycline tolerance in *S. typhimurium*.
Bacteria were grown on plates with varying tetracycline concentrations with no chemical treatment and on plates with tetracycline supplemented with salicylic acid (346 ppm) or Kamba (1827 ppm ae). Results are expressed in EOP ± SEM (n=3). EOPs of the Kamba condition with * are significantly different from the EOPs of the no chemical treatment (p-value < 0.01). EOPs of the salicylic acid condition with # are significantly different to the EOPs of the no chemical condition (p-value < 0.01).

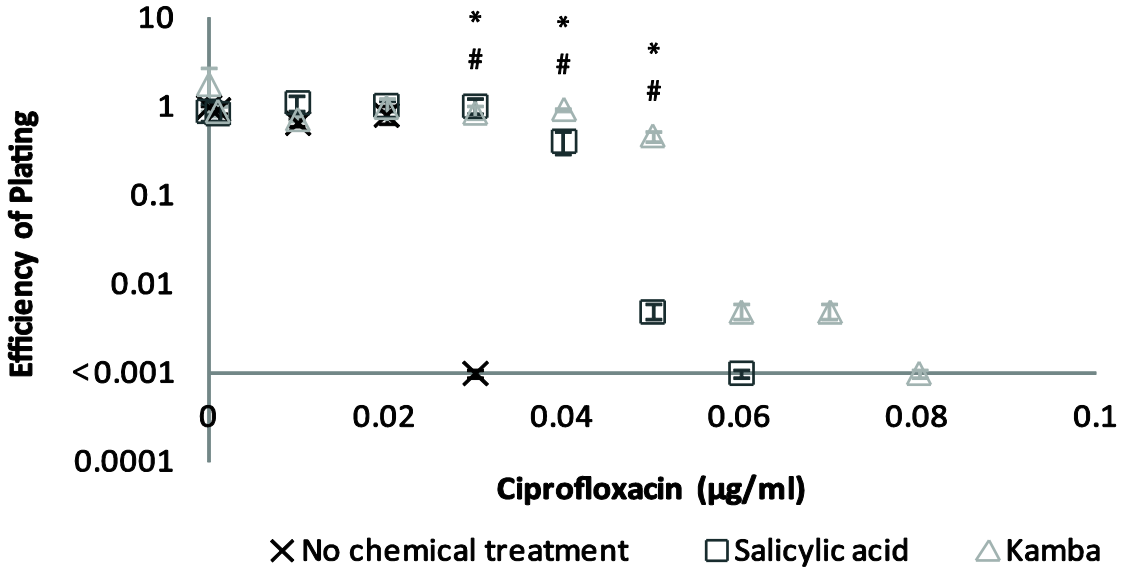

Figure 2.4. Kamba induces ciprofloxacin tolerance in *S. typhimurium*.
Bacteria were grown on plates with varying ciprofloxacin concentrations with no chemical treatment and on plates with ciprofloxacin supplemented with salicylic acid (346 ppm) or Kamba (1827 ppm ae). Results are expressed in EOP ± SEM (n=3). EOPs of the Kamba condition with * are significantly different from the EOPs of the no chemical treatment (p-value < 0.01). EOPs of the salicylic acid condition with # are significantly different to the EOPs of the no chemical condition (p-value < 0.01).

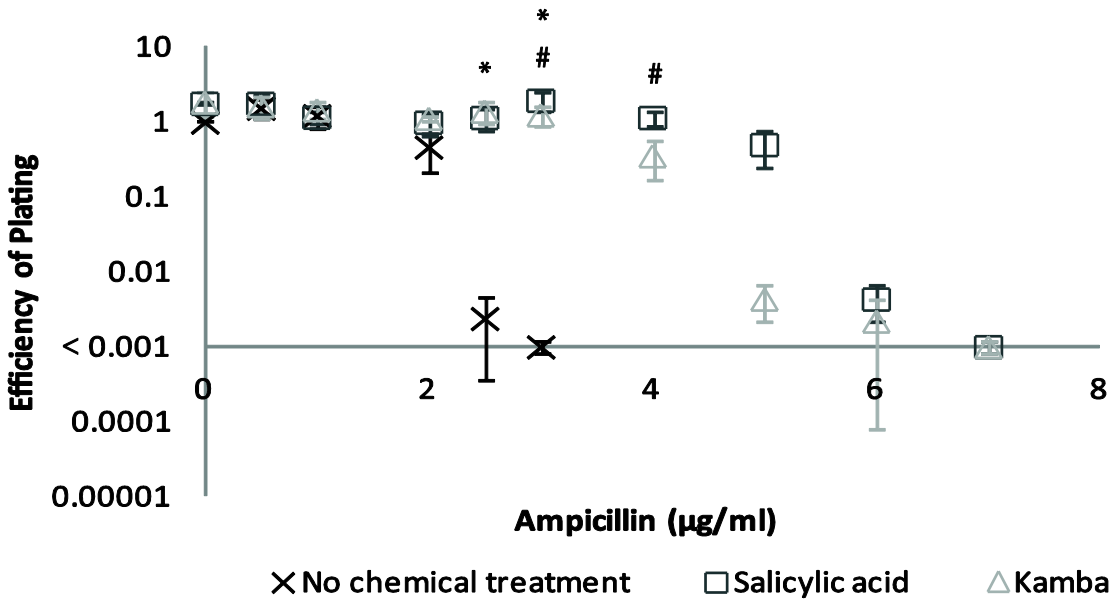

Figure 2.5. Kamba induces ampicillin tolerance in *S. typhimurium*.
Bacteria were grown on plates with varying ampicillin concentrations with no chemical treatment and on plates with ampicillin supplemented with salicylic acid (346 ppm) or Kamba (1827 ppm ae). Results are expressed in EOP ± SEM (n=3). EOPs of the Kamba condition with * are significantly different from the EOPs of the no chemical treatment (p-value < 0.01). EOPs of the salicylic acid condition with # are significantly different to the EOPs of the no chemical condition (p-value < 0.01).

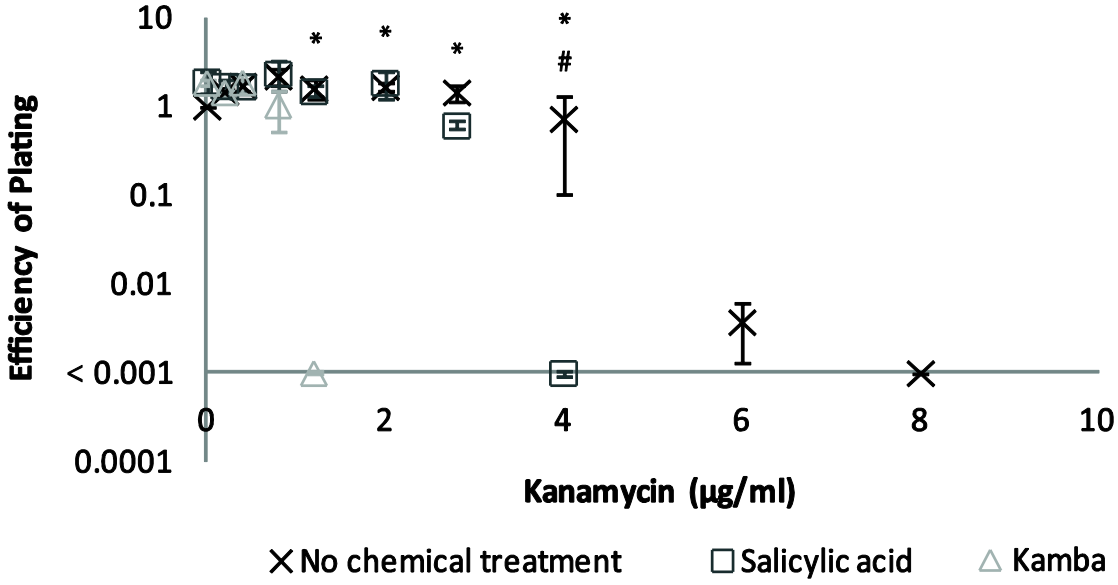

Figure 2.6. Kamba increases kanamycin susceptibility in *S. typhimurium*.
Bacteria were grown on plates with varying kanamycin concentrations with no chemical treatment and on plates with kanamycin supplemented with salicylic acid (346 ppm) or Kamba (1827 ppm ae). Results are expressed in EOP ± SEM (n=3). EOPs of the Kamba condition with * are significantly different from the EOPs of the no chemical treatment (p-value < 0.01). EOPs of the salicylic acid condition with # are significantly different to the EOPs of the no chemical condition (p-value < 0.01).

In contrast to chloramphenicol, tetracycline, ciprofloxacin and ampicillin, Kamba increased sensitivity to kanamycin (Fig. 2.6). Kamba reduced the number of *S. typhimurium* by 0.17-fold (Table 2.7 A). Salicylic acid also caused kanamycin sensitivity, reducing growth by 0.53-fold (Table 2.7 B). This finding is in agreement with previous research which reports that salicylic acid increased kanamycin sensitivity in *E. coli* (Aumercier *et al.*, 1990).

Kamba and salicylic acid had the same effect toward antibiotic response when cultures were grown in liquid media. Cultures exposed to Kamba and salicylic acid had *increased* tolerance to chloramphenicol, tetracycline, ciprofloxacin and ampicillin, but *decreased* kanamycin tolerance (Table 2.8). The ratio of MIC change was generally lower in liquid media compared to growth on solid media.

Table 2.8. Minimum inhibitory concentrations of antibiotics in the presence or absence of chemical treatment measured in liquid media

A

Antibiotic	MIC (µg/ml)		
	(-) Kamba	(+) Kamba	Ratio (+ Kamba/ -Kamba)
Chloramphenicol	6.0 ± 0.0	12.0 ± 0.0	2.00
Tetracycline	2.25 ± 0.00	3.75 ± 0.00	1.67
Ampicillin	8.67 ± 0.67	18.0 ± 0.0	2.08
Ciprofloxacin	0.06 ± 0.00	0.097 ± 0.003	1.62
Kanamycin	10.0 ± 0.0	2.0 ± 0.0	0.20

B

Antibiotic	MIC (µg/ml)		
	(-) Salicylic acid	(+) Salicylic acid	Ratio (+ Salicylic acid/ -Salicylic acid)
Chloramphenicol	6.0 ± 0.0	10.0 ± 0.0	1.67
Tetracycline	2.25 ± 0.00	3.0 ± 0.0	1.33
Ampicillin	8.67 ± 0.67	18.0 ± 0.0	2.08
Ciprofloxacin	0.06 ± 0.00	0.087 ± 0.003	1.45
Kanamycin	10.0 ± 0.0	6.0 ± 0.0	0.60

MICs ± SEM of antibiotics with or without Kamba (A) (1827 ppm ae) and salicylic acid (B) (346 ppm). *S. typhimurium* cultures were grown in liquid LB (24 well plate) and incubated at 37^0C for 16-24 hours.

E. coli grown in the presence of Kamba also had *increased* tolerance to chloramphenicol, tetracycline, ciprofloxacin and *decreased* tolerance to kanamycin (B. Kurenbach and J.A.

Heinemann, personal communication). However, ampicillin tolerance in *E. coli* was unaffected by Kamba, indicating that there are species-specific differences in responses to the herbicides.

2.3.4 2,4-D-induced antibiotic response

The ability of 2,4-D to alter the antibiotic response was examined by plating *S. typhimurium* cultures in the presence of non-toxic concentrations of 2,4-D (1940 ppm ae) and increasing concentrations of each antibiotic. The concentration of 2,4-D used in this assay was below MIC (Table 2.3) and similar to the concentration of Kamba (1827 ppm ae) that caused an antibiotic response. There is slight variation in the exact herbicide concentrations due to different amounts of active ingredients and acid equivalents in the herbicide formulations. There was variation in the number of colonies that grew in the presence of 2,4-D compared to the control with no chemical treatment for all five antibiotics. A summary of the MICs with and without 2,4-D is given below (Table 2.9).

Table 2.9. Minimum inhibitory concentrations of antibiotics in the presence or absence of 2,4-D in solid media

Antibiotic	MIC (µg/ml)		
	(-) 2,4-D	(+) 2,4-D	Ratio (+ 2,4-D/- 2,4-D)
Chloramphenicol	3.44 ± 0.18	6.33 ± 0.33	1.84
Tetracycline	0.78 ± 0.07	1.15 ± 0.22	1.47
Ampicillin	3.50 ± 0.26	8.0 ± 0.0	2.29
Ciprofloxacin	0.03 ± 0.00	0.05 ± 0.00	1.67
Kanamycin	6.89 ± 0.35	4.8 ± 0.0	0.70

MICs ± SEM of antibiotics with or without 2,4-D (1940 ppm ae). *S. typhimurium* cultures were grown on plates and incubated at 37^0C for 16-24 hours.

S. typhimurium exposed to 2,4-D had increased tetracycline, chloramphenicol, ciprofloxacin and ampicillin tolerance. 2,4-D allowed the bacterial cells to grow at chloramphenicol concentrations that are normally lethal (Fig. 2.7). Chloramphenicol MIC was increased 1.84-fold in the presence of 2,4-D (Table 2.9). Similarly, 2,4-D allowed *S. typhimurium* to grow at higher tetracycline concentrations that are normally toxic to cells (Fig. 2.8) which increased the MIC by 1.47-fold (Table 2.9).

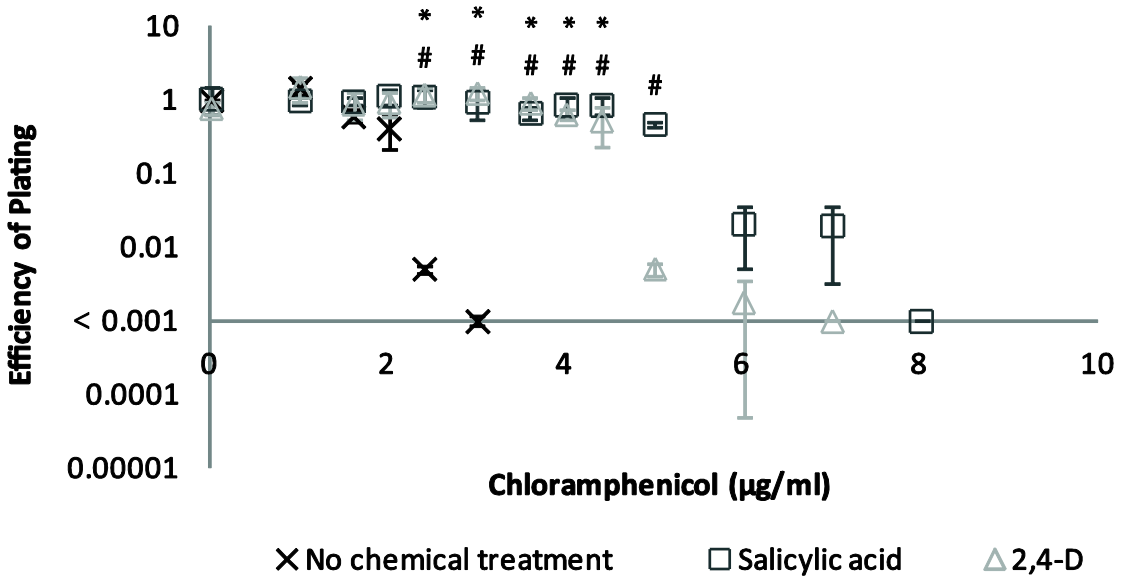

Figure 2.7. 2,4-D induces chloramphenicol tolerance in *S. typhimurium*.
Bacteria were grown on plates with varying chloramphenicol concentrations with no chemical treatment and on plates with chloramphenicol supplemented with salicylic acid (346 ppm) or 2,4-D (1940 ppm ae). Results are expressed in EOP ± SEM (n=3). EOPs of the 2,4-D condition with * are significantly different from the EOPs of the no chemical treatment (p-value < 0.01). EOPs of the salicylic acid condition with # are significantly different to the EOPs of the no chemical condition (p-value < 0.01).

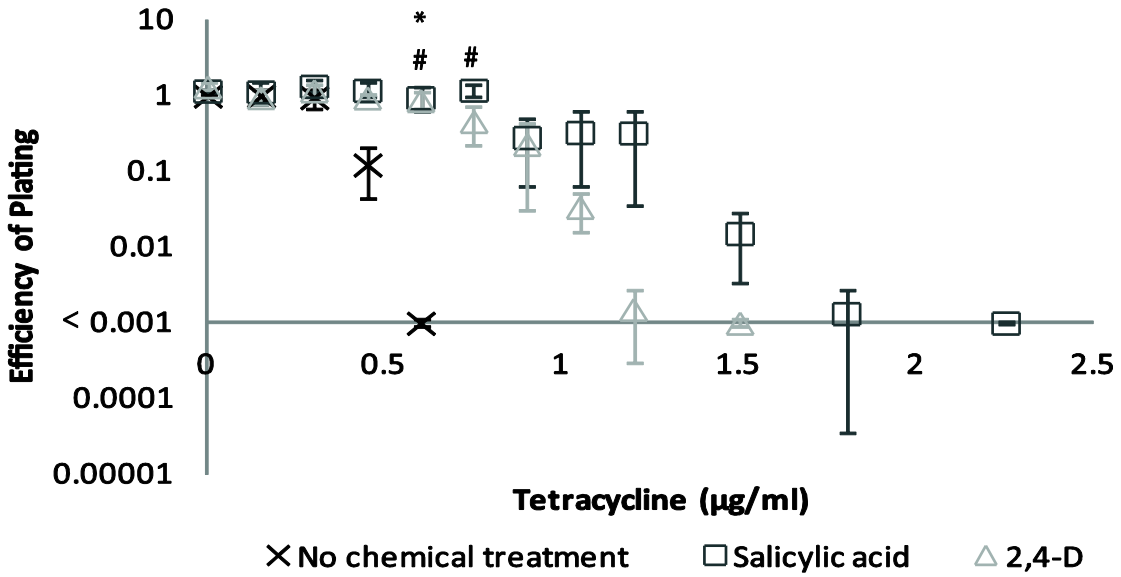

Figure 2.8. 2,4-D induces tetracycline tolerance in *S. typhimurium*.
Bacteria were grown on plates with varying tetracycline concentrations with no chemical treatment and on plates with tetracycline supplemented with salicylic acid (346 ppm) or 2,4-D (1940 ppm ae). Results are expressed in EOP ± SEM (n=3). EOPs of the 2,4-D condition with * are significantly different from the EOPs of the no chemical treatment (p-value < 0.01). EOPs of the salicylic acid condition with # are significantly different to the EOPs of the no chemical condition (p-value < 0.01).

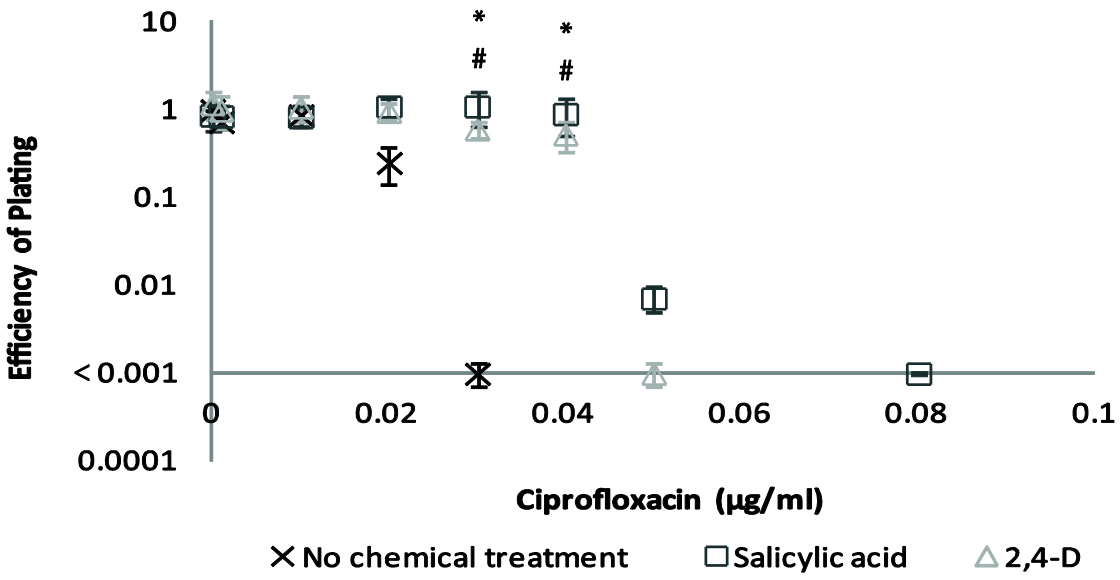

Figure 2.9. 2,4-D induces ciprofloxacin tolerance in *S. typhimurium*.
Bacteria were grown on plates with varying ciprofloxacin concentrations with no chemical treatment and on plates with ciprofloxacin supplemented with salicylic acid (346 ppm) or 2,4-D (1940 ppm ae). Results are expressed in EOP ± SEM (n=3). EOPs of the 2,4-D condition with * are significantly different from the EOPs of the no chemical treatment (p-value < 0.01). EOPs of the salicylic acid condition with # are significantly different to the EOPs of the no chemical condition (p-value < 0.01).

The antimicrobial effect of ciprofloxacin was reduced in the presence of 2,4-D (Fig. 2.9). When ciprofloxacin was the only additive in the growth media, the MIC was 0.03 µg/ml. However, when both ciprofloxacin and 2,4-D were present, the MIC increased to 0.05 µg/ml, a 1.67-fold increase (Table 2.9). 2,4-D also reduced the potency of ampicillin on *S. typhimurium* (Fig. 2.10), increasing the MIC by 2.29-fold (Table 2.9). It is interesting to note that 2,4-D (1940 ppm ae) was not as strong an inducer of tetracycline, chloramphenicol, ciprofloxacin and ampicillin tolerance as was salicylic acid (346 ppm), even though 2,4-D was more concentrated.

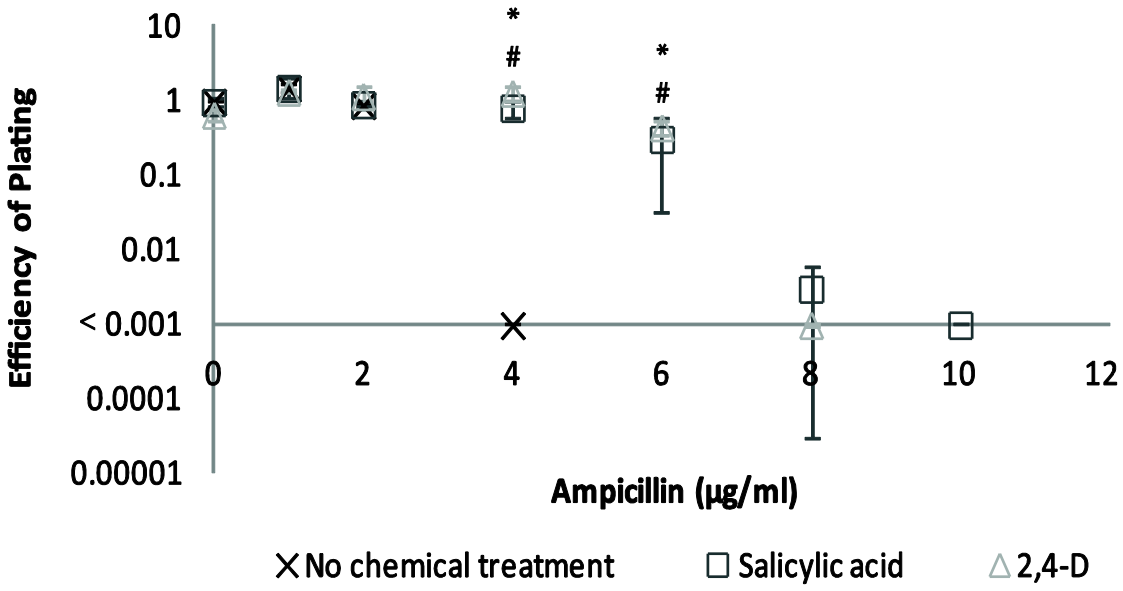

Figure 2.10. 2,4-D induces ampicillin tolerance in *S. typhimurium*.
Bacteria were grown on plates with varying ampicillin concentrations with no chemical treatment and on plates with ampicillin supplemented with salicylic acid (346 ppm) or 2,4-D (1940 ppm ae). Results are expressed in EOP ± SEM (n=3). EOPs of the 2,4-D condition with * are significantly different from the EOPs of the no chemical treatment (p-value < 0.01). EOPs of the salicylic acid condition with # are significantly different to the EOPs of the no chemical condition (p-value < 0.01).

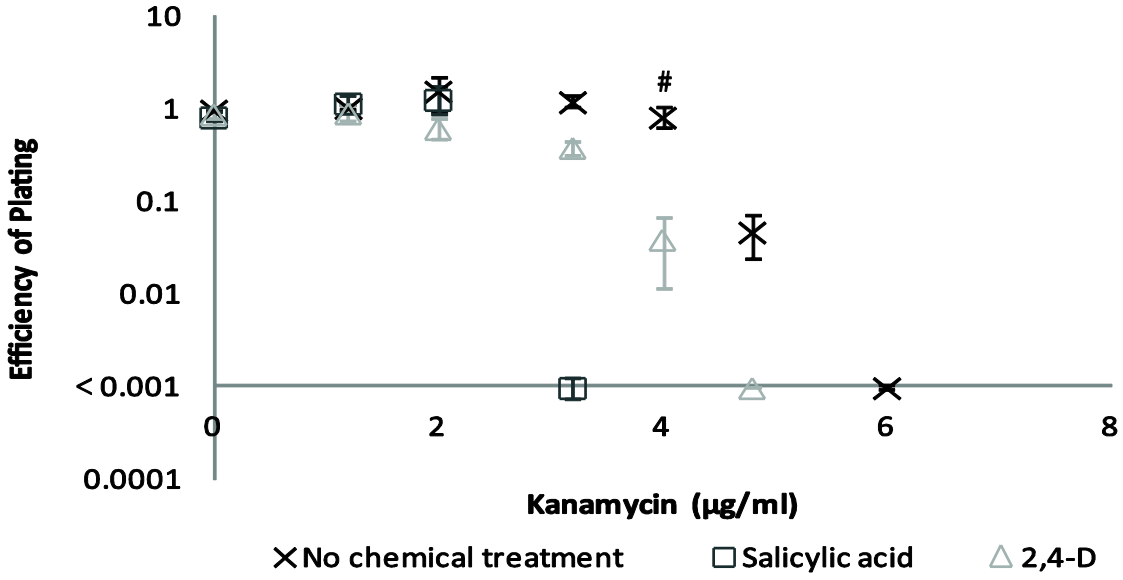

Figure 2.11. 2,4-D increases kanamycin susceptibility in *S. typhimurium*.
Bacteria were grown on plates with varying kanamycin concentrations with no chemical treatment and in plates with kanamycin supplemented with salicylic acid (346 ppm) or 2,4-D (1940 ppm ae). Results are expressed in EOP ± SEM (n=3). EOPs of the salicylic acid condition with # are significantly different to the EOPs of the no chemical condition (p-value < 0.01).

2,4-D increased kanamycin susceptibility in *S. typhimurium*, similar to what was observed with Kamba and salicylic acid. The presence of 2,4-D inhibited bacterial growth at kanamycin concentrations that are normally non-toxic (Fig. 2.11), reducing kanamycin MIC by 0.7-fold (Table 2.9). Overall, salicylic acid and Kamba were stronger inducers of chloramphenicol, tetracycline and ciprofloxacin tolerance and kanamycin susceptibility than 2,4-D (Table 2.7A and 2.9). However, 2,4-D was a stronger inducer of ampicillin tolerance compared to Kamba and salicylic acid.

2,4-D showed the same patterns of antibiotic response in liquid media. It increased *tolerance* toward chloramphenicol, tetracycline, ciprofloxacin and ampicillin and increased *susceptibility* toward kanamycin in *S. typhimurium* (Table 2.10).

Table 2.10. Minimum inhibitory concentrations of antibiotics in the presence or absence of 2,4-D in liquid media

Antibiotic	MIC (μg/ml)		
	(-) 2,4-D	(+) 2,4-D	Ratio (+ 2,4-D/ -2,4-D)
Chloramphenicol	6.0 ± 0.0	8.0 ± 0.0	1.33
Tetracycline	2.25 ± 0.00	3.5 ± 0.3	1.56
Ampicillin	8.67 ± 0.67	16.0 ± 1.0	1.85
Ciprofloxacin	0.06 ± 0.00	0.087 ± 0.003	1.45
Kanamycin	10.0 ± 0.0	5.3 ± 1.3	0.53

MICs ± SEM of antibiotics with or without 2,4-D (1940 ppm ae). *S. typhimurium* cultures were grown in liquid broth (24 well plates) and incubated at 37^0C for 16-24 hours.

2.3.5 Roundup-induced antibiotic response

To study the effects of Roundup on antibiotic tolerance, *S. typhimurium* cultures were plated in the presence of non-toxic concentrations of Roundup (1243 ppm ae) and increasing concentrations of each antibiotic. The concentration of Roundup used in this assay was below MIC and similar to the concentrations of Kamba and 2,4-D that caused an antibiotic response. Roundup showed a different pattern of antibiotic response compared to Kamba and 2,4-D. Bacteria exposed to Roundup showed *increased* tolerance to ciprofloxacin and kanamycin, and *decreased* tolerance to chloramphenicol and tetracycline. A summary of the MICs with and without Roundup is given below (Table 2.11).

Table 2.11. Minimum inhibitory concentrations of antibiotics in the presence or absence of Roundup in solid media

Antibiotic	MIC (μg/ml)		
	(-) Roundup	(+) Roundup	Ratio (+ Roundup/- Roundup)
Chloramphenicol	3.44 ± 0.18	0.35 ± 0.00	0.10
Tetracycline	0.78 ± 0.07	0.35 ± 0.05	0.45
Ampicillin	3.50 ± 0.26	3.33 ± 0.67	0.95
Ciprofloxacin	0.03 ± 0.00	0.17 ± 0.02	5.67
Kanamycin	6.89 ± 0.35	40.0 ± 0.0	5.81

MICs ± SEM of antibiotics with or without Roundup (1243 ppm ae). *S. typhimurium* cultures were grown on plates and incubated at 37^0C for 16-24 hours.

The presence of Roundup inhibited *S. typhimurium* at chloramphenicol concentrations that are normally non-toxic (Fig. 2.12). The MIC of chloramphenicol in the presence of Roundup decreased to 0.35 μg/ml from 3.44 μg/ml (Table 2.11). Roundup increased the antibacterial activity of tetracycline, lowering tetracycline concentrations needed to inhibit *S. typhimurium* (Fig. 2.13). Roundup did not change *S. typhimurium's* susceptibility or tolerance toward ampicillin (Fig. 2.14). The MIC of ampicillin is reduced only marginally, from 3.5 μg/ml without Roundup, to 3.33 μg/ml with Roundup (Table 2.11). However, this difference is within the SEM and was not statistically significant (p-value > 0.01).

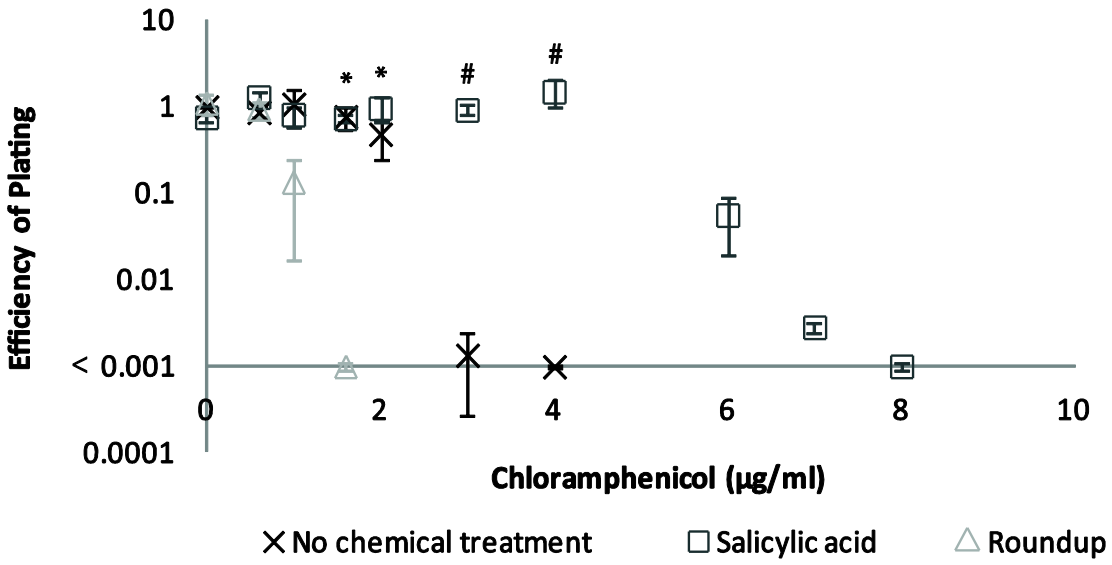

Figure 2.12. Roundup increases chloramphenicol susceptibility in *S. typhimurium*.
Bacteria were grown on plates with varying chloramphenicol concentrations with no chemical treatment and on plates with chloramphenicol supplemented with salicylic acid (346 ppm) or Roundup (1243 ppm ae). Results are expressed in EOP ± SEM (n=3). EOPs of the Roundup condition with * are significantly different from the EOPs of the no chemical treatment (p-value < 0.01). EOPs of the salicylic acid condition with # are significantly different to the EOPs of the no chemical condition (p-value < 0.01).

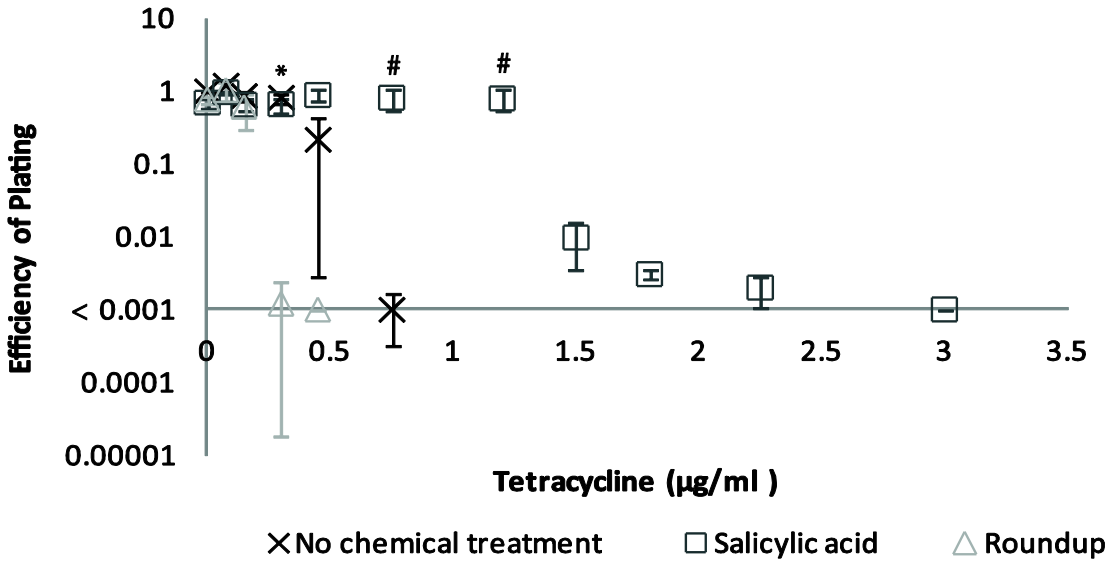

Figure 2.13. Roundup increases tetracycline susceptibility in *S. typhimurium*.
Bacteria were grown on plates with varying tetracycline concentrations with no chemical treatment and on plates with tetracycline supplemented with salicylic acid (346 ppm) or Roundup (1243 ppm ae). Results are expressed in EOP ± SEM (n=3). EOPs of the Roundup condition with * are significantly different from the EOPs of the no chemical treatment (p-value < 0.01). EOPs of the salicylic acid condition with # are significantly different to the EOPs of the no chemical condition (p-value < 0.01).

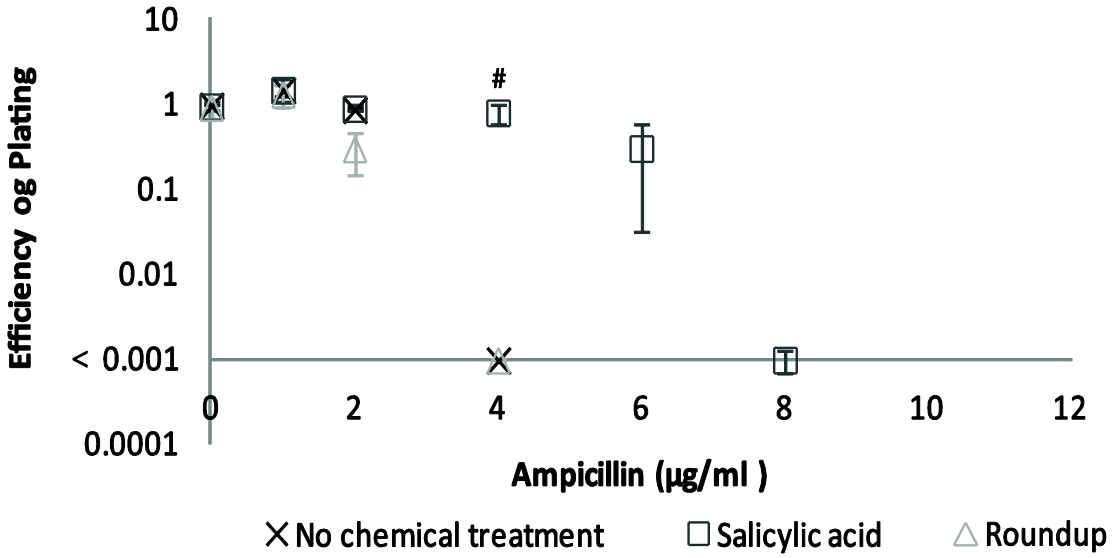

Figure 2.14. Roundup does not affect ampicillin tolerance in *S. typhimurium*.
Bacteria were grown on plates with varying ampicillin concentrations with no chemical treatment and on plates with ampicillin supplemented with salicylic acid (346 ppm) or Roundup (1243 ppm ae). Results are expressed in EOP ± SEM (n=3). EOPs of the salicylic acid condition with # are significantly different to the EOPs of the no chemical condition (p-value < 0.01).

When Roundup was present in the growth media, *S. typhimurium* was able to survive at ciprofloxacin concentrations that would normally inhibit growth (Fig. 2.15). The MIC of ciprofloxacin increased 5.67-fold in the presence of Roundup (Table 2.11).

Roundup enabled the growth of *S. typhimurium* in higher kanamycin concentrations (Fig. 2.16), increasing the MIC by 5.81-fold (Table 2.11). The MIC for kanamycin was 6.89 µg/ml (no chemical treatment), but when media was supplemented with Roundup it increased to 40 µg/ml, which is the concentration used in the laboratory to select kanamycin-resistant strains.

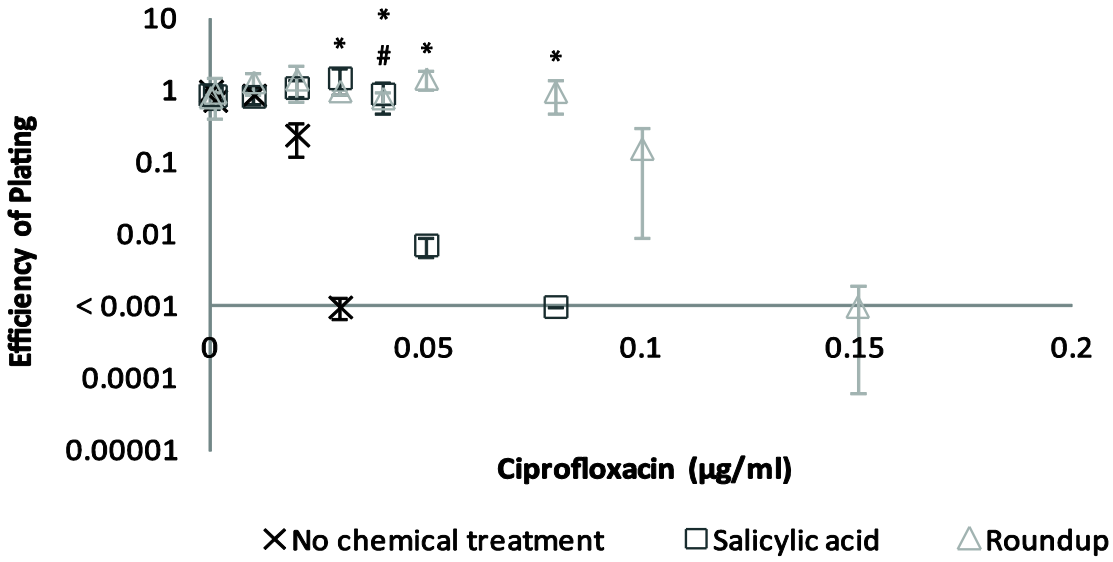

Figure 2.15. Roundup induces ciprofloxacin tolerance in *S. typhimurium*.
Bacteria were grown on plates with varying ciprofloxacin concentrations with no chemical treatment and on plates with ciprofloxacin supplemented with salicylic acid (346 ppm) or Roundup (1243 ppm ae). Results are expressed in EOP ± SEM (n=3). EOPs of the Roundup condition with * are significantly different from the EOPs of the no chemical treatment (p-value < 0.01). EOPs of the salicylic acid condition with # are significantly different to the EOPs of the no chemical condition (p-value < 0.01).

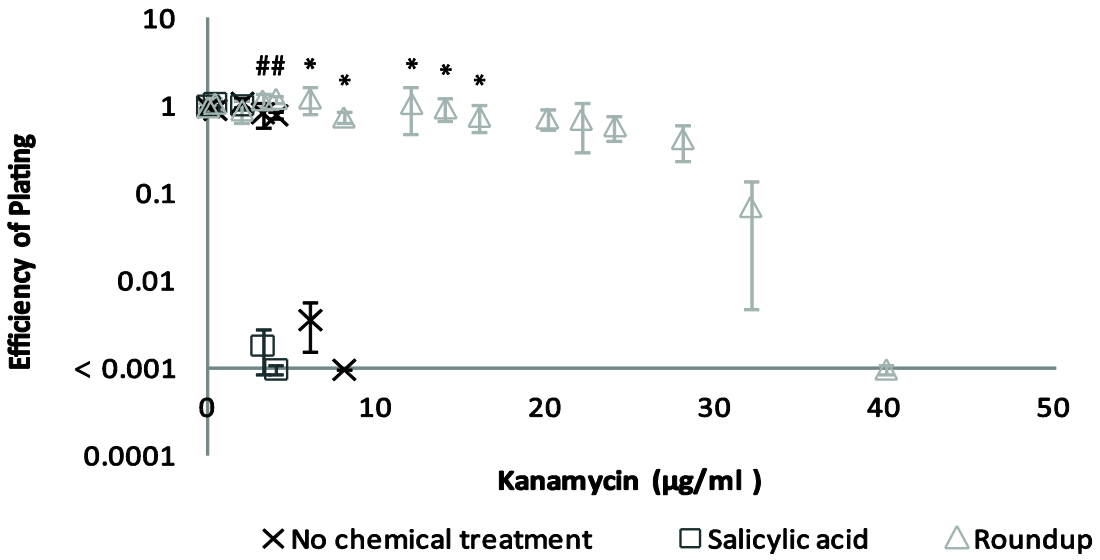

Figure 2.16. Roundup induces kanamycin tolerance in *S. typhimurium*.
Bacteria were grown on plates with varying kanamycin concentrations with no chemical treatment and on plates with kanamycin supplemented with salicylic acid (346 ppm) or Roundup (1243 ppm ae). Results are expressed in EOP ± SEM (n=3). EOPs of the Roundup condition with * are significantly different from the EOPs of the no chemical treatment (p-value < 0.01). EOPs of the salicylic acid condition with # are significantly different to the EOPs of the no chemical condition (p-value < 0.01).

Roundup caused the same pattern of antibiotic tolerance and susceptibility for *S. typhimurium* in liquid media (Table 2.12). It *decreased* the MIC for chloramphenicol and tetracycline by 0.33 and 0.13-fold respectively, while it *increased* the MIC by 2.83 and 4-fold for ciprofloxacin and kanamycin, respectively. In contrast to the results found in solid media, Roundup reduced ampicillin tolerance 0.58-fold.

Table 2.12. Minimum inhibitory concentrations of antibiotics in the presence or absence of Roundup in liquid media

Antibiotic	MIC (µg/ml)		
	(-) Roundup	(+) Roundup	Ratio (+ Roundup/ -Roundup)
Chloramphenicol	6.0 ± 0.0	2.0 ± 0.0	0.33
Tetracycline	2.25 ± 0.00	0.3 ± 0.0	0.13
Ampicillin	8.67 ± 0.67	5.0 ± 0.0	0.58
Ciprofloxacin	0.06 ± 0.00	0.17 ± 0.02	2.83
Kanamycin	10.0 ± 0.0	40.0 ± 0.0	4.00

MICs ± SEM of antibiotics with or without Roundup (1243 ppm ae). *S. typhimurium* cultures were grown in liquid broth (24 well plates) and incubated at 37^0C for 16-24 hours.

2.4 Discussion

Herbicides have become the tool of choice in the war against weeds and their use has increased with the introduction of herbicide-tolerant crops (Benbrook, 2012). Herbicides are not only used in agricultural farming with genetically modified crops but are also used in household gardens for weed control. Therefore, it is important to assess the effect of herbicides on the environment. This study has focused on the effects commercial formulations of dicamba, 2,4-D and glyphosate have on *S. typhimurium*, a pathogenic bacterium. Toxicity levels of these herbicides towards *S. typhimurium* were determined. This study provides evidence of an herbicide-induced (at sub-lethal concentrations) antibiotic response in *S. typhimurium*. In addition, salicylic acid-induced antibiotic tolerance was reconfirmed.

2.4.1 Herbicide toxicity

Herbicides can affect many organisms present in the environment (Bukowska, 2006). The concentrations of Kamba, 2,4-D and Roundup that were toxic to *S. typhimurium* was tested by growing cells in increasing amounts of the herbicides. The lowest concentration that inhibited *S. typhimurium* after overnight (16–20 hours) incubation was taken as the MIC. Results show that 14485 ppm ae Kamba, 5780 ppm ae 2,4-D and 6190 ppm ae Roundup was sufficient to inhibit growth of *S. typhimurium* cultured on solid media. In liquid media, 15737 ppm ae Kamba, 5348 ppm ae 2,4-D and 4897 ppm ae Roundup were capable of inhibiting growth.

The herbicide concentrations that were toxic to *S. typhimurium* were all above the application rates recommended by the manufacturers. Therefore, if farmers adhere to the recommended application rates, the herbicides are unlikely to cause the death of bacteria present on plants and in soil. Herbicide MICs were also above the MRLs for food and feed. Herbicide MRLs, set by Codex Alimentarius, is a global standard that recommends the maximum concentration of pesticide residue that is deemed acceptable *and safe* on crops. Given the results of this study, the concentration of herbicide present in food is unlikely to be lethal to bacteria. However, it should be noted that herbicide MICs were determined for *S. typhimurium* and may be different for other microbes. Lower concentrations of herbicides (but still above application rates) were toxic to *E. coli* (B. Kurenbach and J.A. Heinemann, personal communication).

A study on herbicides found that species richness in aquatic communities was reduced by 22% in the presence of Roundup, while 2,4-D did not have an effect (Relyea, 2005). Herbicide toxicity must be studied in other microbes, using indicator organisms, to give a broader understanding of the MIC of herbicides. In the current study, commercial formulations of herbicides were used. These formulations contain other ingredients, such as surfactants, in addition to the active ingredient. Surfactants are added to herbicides to increase their activity on plants (Liu, 2004). It is unclear if the additives or the active ingredients are toxic to *S. typhimurium*. In order to test this, the MIC of the active ingredient in a pure form has to be determined. This work is currently being undertaken by others.

Soybean and cotton plants that are genetically engineered to tolerate dicamba, are tolerant to seven times the concentrations currently used on most crops (Cao *et al.*, 2011). The availability of such plants will enable farmers to use stronger herbicide concentrations when tolerant weeds appear. For example, the total herbicide application on soybeans was 1.3 kgs/ha in 1996 and by 2006 it increased to 1.6 kgs/ha (Benbrook, 2012). Therefore, herbicide concentrations might reach lethal levels for bacteria.

2.4.2 Effect of herbicides on antibiotic response

The aim of this experiment was to test if exposure to one toxin (at sub-lethal concentrations) causes an increase in tolerance to other toxins. To test the effect of herbicides on antibiotic tolerance, *S. typhimurium* was grown in the presence of an herbicide and different concentrations of antibiotic in solid and liquid media. The effect of each herbicide, on solid media, was determined by the number of colonies that grew in each treatment. This bacterial growth was converted to EOP, which standardises growth to the control that is not treated with herbicide or antibiotic. Three commercial formulations of herbicides: Kamba, 2,4-D and Roundup, and five antibiotics: chloramphenicol, tetracycline, ciprofloxacin, ampicillin and kanamycin were tested. *S. typhimurium* exposed to herbicides had altered antibiotic response.

Exposure to Kamba was sufficient to increase the MIC of chloramphenicol, tetracycline, ciprofloxacin and ampicillin. *S. typhimurium* exposed to Kamba showed increased kanamycin

susceptibility. 2,4-D also induced chloramphenicol, tetracycline, ciprofloxacin, ampicillin tolerance and induced kanamycin susceptibility. However, the magnitude of the antibiotic response varied between the two herbicides. Kamba was a stronger inducer of chloramphenicol, tetracycline, and ciprofloxacin tolerance and kanamycin susceptibility, while 2,4-D was a stronger inducer of ampicillin tolerance. This suggests that the Kamba formulation has a greater effect on mechanisms that cause chloramphenicol, tetracycline and ciprofloxacin tolerance.

Except kanamycin, salicylic acid induced tolerance to the other antibiotics tested in *S. typhimurium*. The concentration of salicylic acid used in this experiment was lower than the herbicide concentrations, and it was a stronger inducer of antibiotic tolerance than 2,4-D. It is unclear if the structural similarity of 2,4-D and Kamba to salicylic acid explains the similar responses that they induce. Nevertheless, pure salicylic acid is capable of causing the effect so the additional ingredients in the formulation are probably not necessary for the effect, but they may influence the magnitude of the effect.

Roundup induced ciprofloxacin and kanamycin tolerance while it increased chloramphenicol and tetracycline susceptibility. The strongest inducer of antibiotic tolerance was Roundup towards kanamycin and ciprofloxacin. The MIC increased 5.67 and 5.81-fold, respectively. The level of kanamycin tolerance (up to 40 µg/ml) induced by Roundup was close to the tolerances seen by kanamycin tolerant strains. Roundup seems to have little effect on ampicillin tolerance. As mentioned earlier, the effect of salicylic acid seen in this experiment is in agreement with other studies (Rosner, 1985; Aumercier *et al.*, 1990; Berlanga & Vinas, 2000).

The antibiotic response induced by the herbicides in the current experiment was ‘immediate’. A pre-incubation step with the herbicides prior to antibiotic exposure was not required. This is to say that the herbicide reduced susceptibility to the antibiotics faster than the antibiotics could kill or inhibit the cell. This is especially interesting as at least three of the antibiotics block gene expression. In the study conducted by Rosner (1985) the effect of salicylic acid on

chloramphenicol tolerance was also immediate (Rosner, 1985). This indicates that the herbicides may be affecting antibiotic tolerance through similar pathways as salicylic acid.

E. coli grown in the presence of salicylic acid have been shown to have increased production of the multidrug efflux pump AcrAB-TolC (Price *et al.*, 2000). In addition, salicylic acid also reduces the amount of OmpF (an outer membrane porin) (Price *et al.*, 2000). This change in membrane proteins reduces the influx of antibiotics and increases the efflux of intracellular drugs, resulting in tolerance (Price *et al.*, 2000). The antibiotic response induced by Kamba, 2,4-D and Roundup may be due to changes in the amounts of membrane porins and efflux pumps. However, the down regulation or up-regulation of membrane pumps by Kamba, 2,4-D and salicylic acid appears to be different to Roundup. It is well known that different compounds induce specific and different patterns of tolerance and susceptibility. Given the chemical differences of glyphosate (Fig. 1.1), this is to be expected. The possible changes in membrane proteins caused by these herbicides raise concerns as it can give rise to antibiotic tolerant strains, compromising the treatment of bacterial infections.

The antibiotics tested in this study were all clinically relevant antibiotics that are used in the treatment of infectious diseases. For example, kanamycin is used in the treatment of *Mycobacterium tuberculosis* infections (Reeves *et al.*, 2013), and ciprofloxacin is used to treat typhoid infections (Lee *et al.*, 2013) and urinary tract infections (Arslan *et al.*, 2005). The emergence of tolerant strains reduces the effectiveness of antibiotics in the fight against infectious diseases. Strains that are tolerant to specific antibiotics are known to become tolerant to other antibiotics. These strains are referred to as multidrug resistant (MDR) strains (Piddock, 2006). MDR strains that have increased expression of a particular efflux pump will have increased tolerance to all antibiotics that can pass through it (Fernandez & Hancock, 2012). Therefore, tolerances induced by the herbicides could make strains resistant to other antibiotics.

Although the herbicides tested in this study caused low level antibiotic tolerance, it is still relevant as strains that have low level tolerance may develop a gradual increase in tolerance

(Fernandez & Hancock, 2012). This could eventually result in high level tolerance (Piddock, 2006; Fernandez & Hancock, 2012). These relatively minor increases in MICs is referred to as the 'MIC creep' (Steinkraus *et al.*, 2007). Following the introduction of antibiotics in therapeutics, there has been a steady increase in antibiotic tolerance and MIC increase worldwide, compromising their use against pathogenic bacteria (Fernandez *et al.*, 2011).

A study that surveyed antibiotic tolerance over a 8 year period found slow but steady increase in ciprofloxacin tolerance (Oudhuis *et al.*, 2008). Furthermore, there was an increase in the number of antibiotics to which multi-tolerant isolates developed tolerance (Oudhuis *et al.*, 2008). The increase in antibiotic MICs can cause a significant increase in treatment failure. Vancomycin is an antibiotic used to treat patients with methicillin-tolerant *Staphylococcus aureus* infections (Lodise *et al.*, 2008). However, the effectiveness of this antibiotic was dependent on the MIC of the infecting bacteria. Patients infected with populations that have high vancomycin MICs (greater than or equal to 1.5 mg/L) had a 2.4-fold increase in treatment failure compared to patients infected with populations with lower MICs (less than 1.5mg/L) (Lodise *et al.*, 2008). Similarly, patients treated with ofloxacin for *S. typhimurium* infections, had significantly higher treatment failure when infections had high MIC compared to infections with low MIC (Parry *et al.*, 2011).

The European Committee on Antimicrobial Susceptibility Testing has suggested that *S. typhimurium* with a ciprofloxacin MIC greater than 0.06 μg/ml is likely to result in treatment failure (Hassing *et al.*, 2013). Here, *S. typhimurium* grown in the presence of Kamba and Roundup had tolerance up to 0.08 μg/ml and 0.17 μg/ml ciprofloxacin, respectively (Table 2.7 and 2.11). Therefore, the increase in ciprofloxacin tolerance caused by Kamba and Roundup may compromise treatment success. In addition, the protection caused by the herbicides against antibiotics may also allow the accumulation of mutations that may give rise to high level tolerance, further increasing treatment failure (Piddock, 2006; Fernandez & Hancock, 2012).

Due to increased treatment failure, the dosage of antibiotics may have to be increased or other antibiotics used (Haeseker *et al.*, 2013; Hassing *et al.*, 2013). A study conducted by Haeseker *et*

al. (2013) recommended increasing the standard dose of ciprofloxacin to 1200 mg day^{-1} (from 800 mg day^{-1}) for effective treatment (Haeseker *et al.*, 2013). However, use of antibiotics at high dosages may increase the chance of adverse drug events (Haeseker *et al.*, 2013). The concentrations of herbicides that induced antibiotic tolerance here were above MRLs, but within the application rate concentrations. Therefore, microbes on plants or in soil which are likely to be exposed to high herbicide concentrations could develop increased antibiotic tolerance. As I will describe in a later chapter, much lower post-induction concentrations may be sufficient to maintain tolerance.

Chapter Three

3. Minimum Herbicide Concentrations that Induce an Antibiotic Response

3.1 Introduction

The previous experiments (Chapter 2) conducted as part of this study provided evidence of an herbicide-induced antibiotic response. The herbicide concentrations used in those experiments were above Maximum Residue Limits (MRLs) set for food and feed. This prompted the investigation of whether lower herbicide concentrations also induce an antibiotic response. *Salmonella enterica* serovar *Typhimurium* was grown in the presence of antibiotics and decreasing concentrations of herbicides. The lowest herbicide concentration that increased Efficiency Of Plating (EOP) by 100-fold compared to exposure only to the antibiotic was taken as the "lowest induction concentration". The minimum inducing concentrations of Kamba, 2,4-D and Roundup were determined for the five antibiotics: chloramphenicol, tetracycline, ciprofloxacin, ampicillin and kanamycin.

As part of this study, the effect of Kamba on chloramphenicol tolerance was examined in tap water. *S. typhimurium* was first grown in tap water with Kamba or Kamba with chloramphenicol. The cultures were then plated on chloramphenicol plates (above Minimum Inhibitory Concentration (MIC)) to determine the number of *S. typhimurium* tolerant to chloramphenicol.

Increased herbicide use has raised concerns about herbicide residues in the environment, having been found in rivers, streams and food products (Shipitalo *et al.*, 2008) which present potential hazards for ecosystems and human health (Soloneski & Larramendy, 2011). International agencies, such as Codex Alimentarius, have prescribed MRLs for herbicides. Dicamba, 2,4-D and glyphosate residues have been found in various environments and food

products such as oats, bread and cereals, but at low concentrations (below MRLs) (Center for Food Safety and Applied Nutrition - U.S. Food and Drug Administration, 2004; Benbrook, 2012).

How these MRLs, or other pathways of exposure, translate into levels of herbicide in humans or animals is poorly understood. However, there are a growing number of reports that the cumulative exposure to some herbicides is leading to high *in vivo* levels. A grassroots campaign conducted by "Moms Across America" on drinking water, breast milk and urine samples volunteered from across the U.S. found glyphosate levels in breast milk as high as 76 ppb to 166 ppb, 0.085 ppb and 0.33 ppb in drinking water, and 18.8 ppb in urine (Moms Across America, 2014). The peak concentrations in bodily fluids were higher than the allowable exposure from food or water, but below what American regulatory agencies consider to be toxic to people.

An European study of farm animals and humans found glyphosate levels varied by country and proximity to pesticide use practices, such as using glyphosate as part of a post-harvest desiccation process (Krüger *et al.*, 2014). This study also reported a statistically significant correlation between *in vivo* glyphosate levels and chronic disease state. The levels detected were also in the ppb range.

The application rates of herbicides are higher than MRLs. These are the concentrations to which environmental bacteria are exposed. These bacteria may be simultaneously exposed to antibiotics via insect vectors. For example, honeybees may visit recently sprayed crops and return to hives being treated with tetracycline (Tian *et al.*, 2012a). The practice in the U.S. has resulted in a high rate of carriage of antibiotic tolerance in American bees with the genes coming from species that include human pathogens (Tian *et al.*, 2012a). This indicates the potential for mingling of microbes through bees. Furthermore, houseflies carry bacteria from farm animal faeces into homes (Zurek & Ghosh, 2014). Farm animal faeces and urine are sources of bacteria exposed to both herbicides (Krüger *et al.*, 2013; Krüger *et al.*, 2014) and antibiotics (Joy *et al.*, 2013), the latter at potentially sub-lethal concentrations.

Continued herbicide use in agriculture can cause residue accumulation that may contaminate surface and ground water through drift, runoff, drainage and leaching (Cerejeira *et al.*, 2003).

Potential contamination of ground water is alarming because in many countries it is a source of drinking water (Cerejeira *et al.*, 2003), directly exposing humans to herbicides (Hamilton *et al.*, 2003). Guidelines for Maximum Contamination Levels (MCLs) in water have been set by national governments and international agencies such as the U.S. Environmental Protection Agency (EPA) (Hamilton *et al.*, 2003). Residues of dicamba and 2,4-D have been found in agricultural and urban runoffs (Kuo *et al.*, 2012; Ensminger *et al.*, 2013). Furthermore, in areas where crops are extensively grown, herbicides have been detected at concentrations above the MCL (Shipitalo *et al.*, 2008). Hence, herbicides present in water environments may affect bacteria and their antibiotic response.

3.2 Methods

3.2.1 Bacterial strain, growth conditions and chemicals

Bacterial cultures were grown and maintained as described in Chapter 2 (section2.2.1). All assays were done with *Salmonella enterica* serovar *Typhimurium* SL3770 (*rfa*$^{+}$) (MacLachlan & Sanderson, 1985), and the three commercial herbicides: Kamba, 2,4-D and Roundup.

3.2.2 Determining minimum herbicide concentrations that induce an antibiotic response

An assay was performed to determine if and at what concentrations of herbicides, lower than those used in Chapter 2, induced an antibiotic response. *S. typhimurium* was grown in Luria Broth (LB) media at 37^{0}C until the optical density (OD_{600}) reached 1. Then the culture was diluted 10-fold in LB media. Three dilutions were plated in duplicate on plates with antibiotic (equal to or above MIC for tolerance, below MIC for susceptibility) and increasing concentrations of herbicide. The plates were allowed to dry and were then incubated at 37^{0}C for 16-24 hours. All three herbicides (Kamba, 2,4-D and Roundup) were tested with the five antibiotics: chloramphenicol, tetracycline, ampicillin, ciprofloxacin and kanamycin. Controls included plates with antibiotic (no herbicide) and no chemical treatment (no herbicide or antibiotic).

Titres were determined for cultures in each treatment and the Efficiencies Of Plating (EOP) were calculated (as described in Chapter 2, section 2.2.3.1). An antibiotic response that varied the EOP by a factor of 100 was taken as an arbitrary but stringent threshold. The lowest herbicide concentration that sustained a viable population above this threshold was taken as the minimum induction concentration for antibiotic tolerance. For herbicides that caused increased antibiotic susceptibility, the lowest concentration that reduced the viable population to below the threshold was taken as the minimum induction concentration.

3.2.3 Determining if antibiotic response is induced by herbicides in tap water

To determine if an antibiotic response could be observed when bacteria were exposed to herbicides in water, *S. typhimurium* was grown in LB till the OD_{600} reached 1. Then the bacterial cells (from 5ml of culture) were harvested from LB by centrifugation at 6613 x *g* for 1 min (Fig. 3.1). The supernatant was discarded and the cell pellet was re-suspended in 5 ml filter-sterilized tap water. The tap water used in this experiment was filtered through a 0.2 µm Supor membrane low protein binding non pyrogenic filter. Culture samples of 1 ml were then incubated in the following conditions: tap water, tap water with 10% LB plus Kamba (1827 ppm ae), tap water with 10% LB plus chloramphenicol (4 µg/ml) and Kamba (1827 ppm ae). In addition, tap water without the bacterial culture was included as a blank. The total volume of each condition was 10 ml.

To determine the number of *S. typhimurium* in each treatment before incubation (Time = 0), aliquots from each condition were taken and serially diluted in LB. Three dilutions were then plated on LB and LB with chloramphenicol (4 µg/ml) in duplicate. The plates and cultures were incubated for 16-20 hours at 37^0C, liquid cultures were incubated in a Thermolyne-ROSI 1000 orbital shaking incubator at 185 rpm.

Following incubation (16-20 hours), the liquid cultures were serially diluted in LB and plated on LB and LB with chloramphenicol (4 µg/ml). The plates were incubated for 16-20 hours at 37^0C. The titre was determined for cultures grown in each condition before (Time = 0 hrs) and after (Time = 16-20 hours) incubation. The difference in the number of colony forming units, before and after incubation, was calculated. This assay was done for Kamba and chloramphenicol only.

3.2.4 Statistical analysis

Using the program R, the data gathered from the minimum induction concentration assay and the tap water assay, was tested for statistical difference between treatments. The Kruskal-Wallis test, a non-parametric analysis of variance, was used for both assays because the data was not normally distributed. This method tests if there is a difference between

treatments but does not identify which treatment is different from the others (Elliott & Hynan, 2011) For the tap water assay, the cfu/ml from chloramphenicol plates were compared with each treatment. The p-value threshold was set at < 0.01, meaning that the likelihood of the observed result occurred by chance alone is less than 1%. For the Kruskal-Wallis test, a significant result means that at least one of the treatments is significantly different from the other treatments, therefore, a p-value below 0.01 was taken as statistically significant.

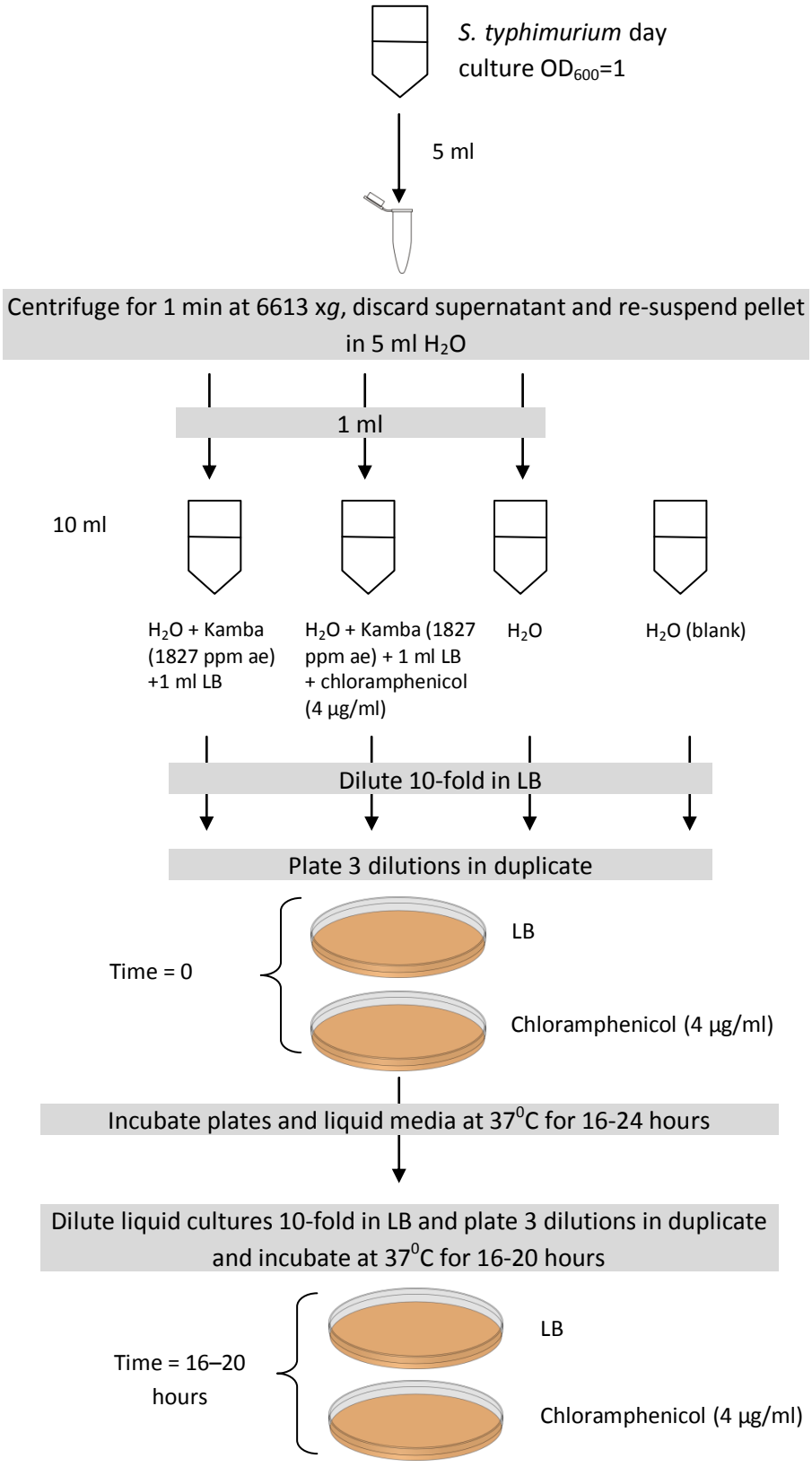

Figure 3.1. Schematic diagram of the tap water experiment.

3.3 Results

3.3.1 Effect of various Kamba concentrations on antibiotic response

To determine whether the concentration of Kamba affects the level of antibiotic response induced by the herbicide, *S. typhimurium* was grown in antibiotics supplemented with various amounts of Kamba. Antibiotic concentrations were above MIC when bacteria were exposed to herbicides that increase tolerance to a particular antibiotic and to concentrations below MIC when the herbicide induced greater sensitivity to the antibiotics. There was a small degree of day-to-day variation in the MIC. Following incubation, the number of colonies that grew in each treatment were counted and converted to a measure of EOP. A differential and conservative threshold was set at 100x EOP of the antibiotic-only treatment to determine the lowest herbicide concentration at which a large proportion of *S. typhimurium* remained viable in the presence of antibiotic. The lowest Kamba concentration that enabled *S. typhimurium* growth above or below the threshold was taken as the minimum inducing concentration for antibiotic tolerance or susceptibility, respectively.

Kamba concentration positively correlated with the titre of chloramphenicol (Fig. 3.2), tetracycline (Fig. 3.3), ciprofloxacin (Fig. 3.4) and ampicillin (Fig. 3.5) tolerant colonies. In contrast, Kamba concentration negatively correlated with the titre of kanamycin tolerant colonies (Fig. 3.6). Therefore, Kamba's ability to cause an antibiotic response was concentration-dependent. The lowest concentration of Kamba that was capable of inducing antibiotic tolerance varied with the antibiotic, ranging from 243.7 ppm ae to 426.4 ppm ae (Table 3.1). Statistical analysis showed that at least one treatment significantly varied from the other treatments at the p-value threshold of less than 0.01 for all antibiotics. Kamba concentrations as low as 137.1 ppm ae were sufficient to induce kanamycin susceptibility. The inducing concentrations determined here were within the recommended application rate (Table 2.6) but above the MRLs for dicamba (Table 2.5).

Table 3.1. Minimum Kamba concentrations that induced an antibiotic response

Antibiotic	Kamba (ppm ae)
Chloramphenicol	274.1 ± 52.8
Tetracycline	426.4 ± 60.9
Ciprofloxacin	243.7 ± 30.5
Ampicillin	303.9 ± 30.8
Kanamycin[1]	137.1 ± 0.0

Minimum Kamba concentrations ± SEM that cause antibiotic tolerance or susceptibility[1] in *S. typhimurium* were measured on plates and grown at 37^0C for 16-24 hours. Kamba concentrations are expressed in ppm ae.

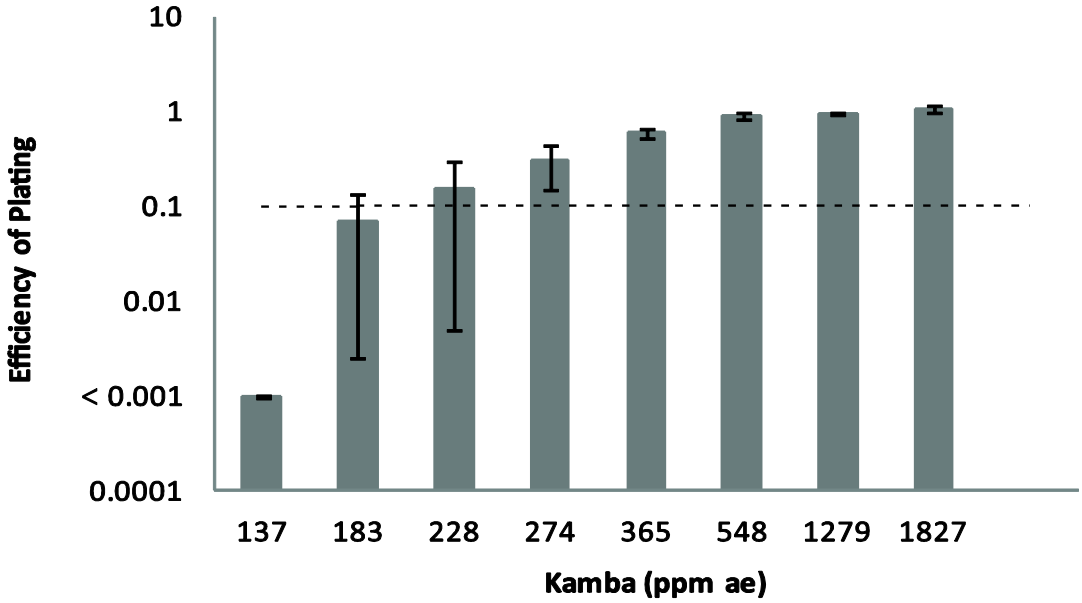

Figure 3.2. Kamba concentration-dependent chloramphenicol tolerance in *S. typhimurium*.
Bacteria were grown on plates with chloramphenicol (4 μg/ml) supplemented with varying amounts of Kamba. Results are expressed in EOP ± SEM; the dotted line represents the differential threshold. At least one treatment is statistically significant from the other treatments (p-value = 0.00046, by Kruskal-Wallis test).

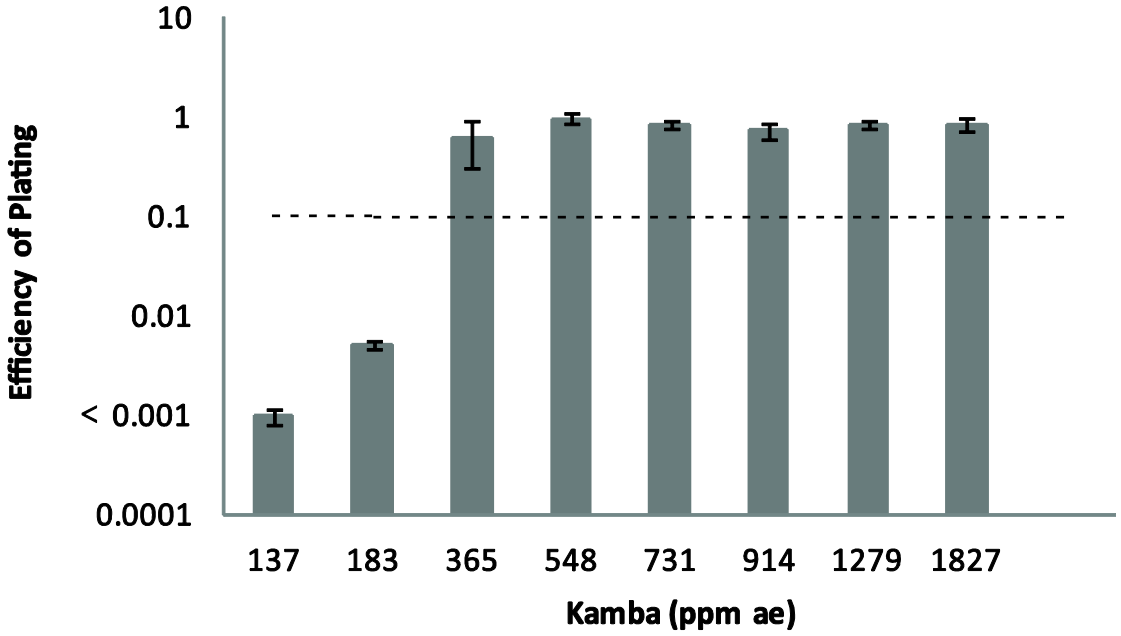

Figure 3.3. Kamba concentration-dependent tetracycline tolerance in *S. typhimurium*.
Bacteria were grown on plates with tetracycline (0.75 µg/ml) supplemented with various amounts of Kamba. Results are expressed in EOP ± SEM; the dotted line represents the differential threshold. At least one treatment is statistically significant from the other treatments (p-value = 0.00064, by Kruskal-Wallis test).

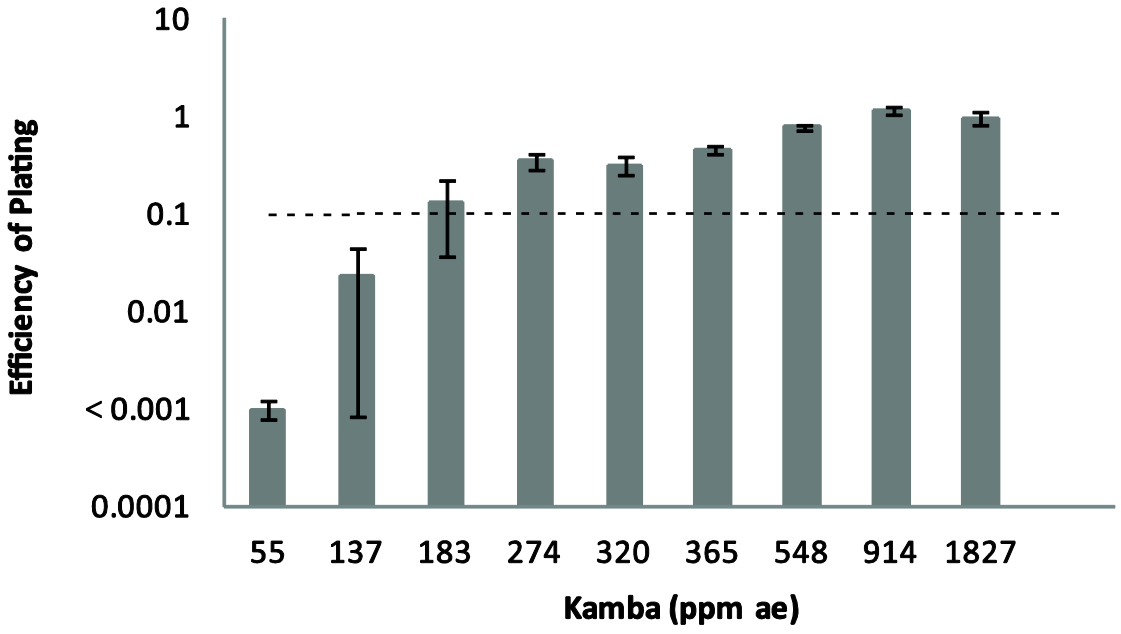

Figure 3.4. Kamba concentration-dependent ciprofloxacin tolerance in *S. typhimurium*.
Bacteria were grown on plates with ciprofloxacin (0.03 µg/ml) supplemented with various amounts of Kamba. Results are expressed in EOP ± SEM; the dotted line represents the differential threshold. At least one treatment is statistically significant from the other treatments (p-value = 0.000071, by Kruskal-Wallis test).

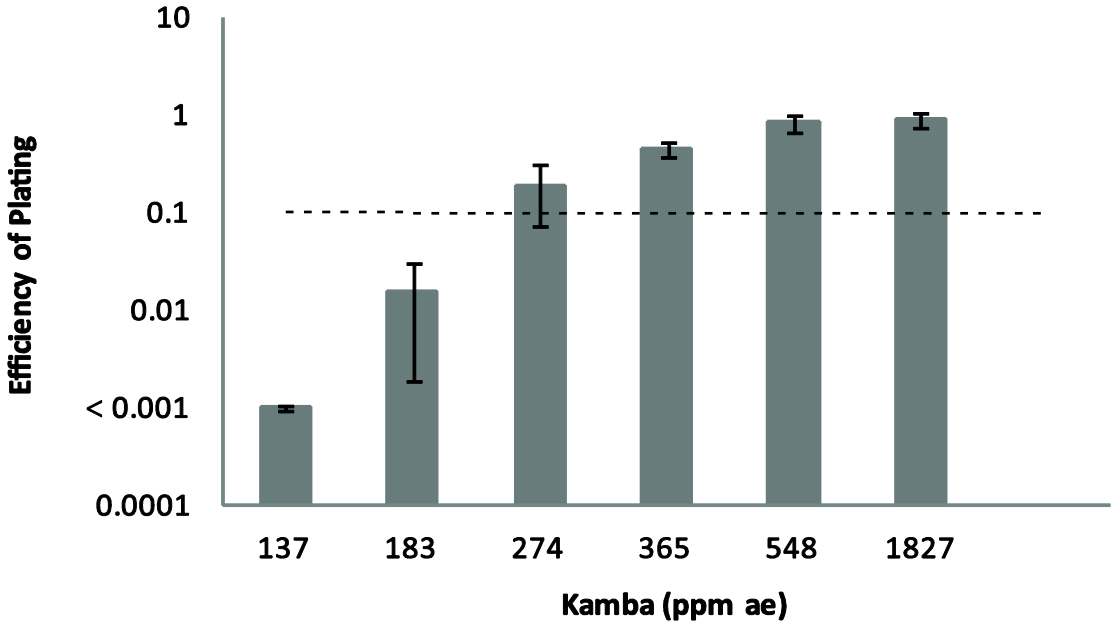

Figure 3.5. Kamba concentration-dependent ampicillin tolerance in *S. typhimurium*.
Bacteria were grown on plates with ampicillin (2 µg/ml) supplemented with various amounts of Kamba. Results are expressed in EOP ± SEM; the dotted line represents the differential threshold. At least one treatment is statistically significant from the other treatments (p-value = 0.00045, by Kruskal-Wallis test).

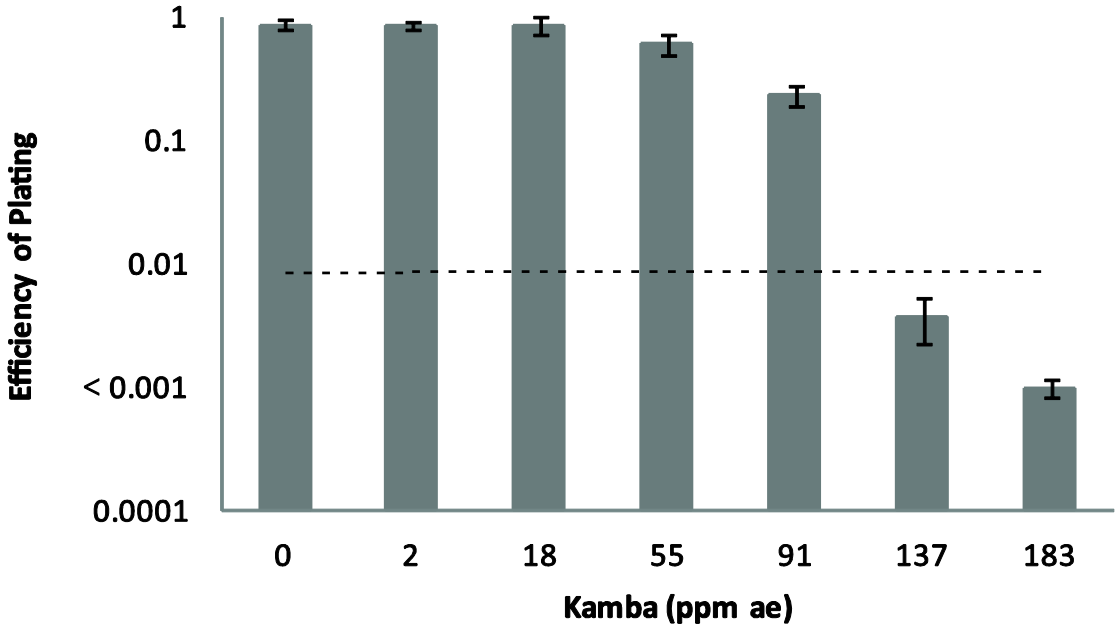

Figure 3.6. Kamba concentration-dependent kanamycin susceptability in *S. typhimurium*.
Bacteria were grown on plates with kanamycin (2 µg/ml) supplemented with various amounts of Kamba. Results are expressed in EOP ± SEM; the dotted line represents the differential threshold. At least one treatment is statistically significant from the other treatments (p-value = 0.0014, by Kruskal-Wallis test).

3.3.2 Effect of various 2,4-D concentrations on antibiotic response

S. typhimurium was grown in antibiotics supplemented with decreasing amounts of 2,4-D to determine the lowest concentration that induced an antibiotic response. Antibiotic concentrations were above MIC when bacteria were exposed to herbicides that increase tolerance to a particular antibiotic and to concentrations below MIC when the herbicide induced greater sensitivity to the antibiotic. Similar to the assay on Kamba, a differential and conservative threshold was set at 100x EOP of the antibiotic-only treatment. The lowest 2,4-D concentration that enabled growth above or below the threshold was taken as the minimum inducing concentration for antibiotic tolerance or susceptibility, respectively.

2,4-D concentration positively correlated with the titres of chloramphenicol (Fig. 3.7), tetracycline (Fig. 3.8), ciprofloxacin (Fig. 3.9) and ampicillin (Fig. 3.10) tolerant *S. typhimurium*. While 2,4-D concentration negatively correlated with the titres of kanamycin tolerant *S. typhimurium* (Fig. 3.11). Similar to Kamba, the minimum 2,4-D concentrations that had an effect on response varied with the antibiotic. Moreover, there was a wide variation in minimum inducing concentrations. For example, 19.4 ppm ae 2,4-D induced tetracycline tolerance, whereas, 1009.5 ppm ae was necessary to induce ciprofloxacin tolerance (Table 3.2). Statistical analysis of the data showed that at least one treatment was statistically different from the other treatments for chloramphenicol (p-value = 0.002), ciprofloxacin (0.001) and kanamycin (0.0038). At the 0.01 p-value threshold, the treatments for tetracycline (p-value = 0.019) and ampicillin (p-value = 0.013) were only marginally significant. The concentrations that affected antibiotic response were within the recommended application rates (Table 2.6). *Furthermore, the minimum concentration that induced tetracycline, chloramphenicol and ampicillin tolerance were at or below the MRL range* (Table 2.5).

Table 3.2. Minimum 2,4-D concentrations that induced an antibiotic response

Antibiotic	2,4-D (ppm ae)
Chloramphenicol	355.7 ± 32.3
Tetracycline	19.4 ± 0.0
Ciprofloxacin	1009.5 ± 129.3
Ampicillin	258.7 ± 32.3
Kanamycin[1]	2522.3 ± 194.1

Minimum 2,4-D concentrations ± SEM that cause antibiotic tolerance or susceptibility[1] in *S. typhimurium* were measured on plates and grown at 37^0C for 16-24 hours. 2,4-D concentrations are expressed in ppm ae.

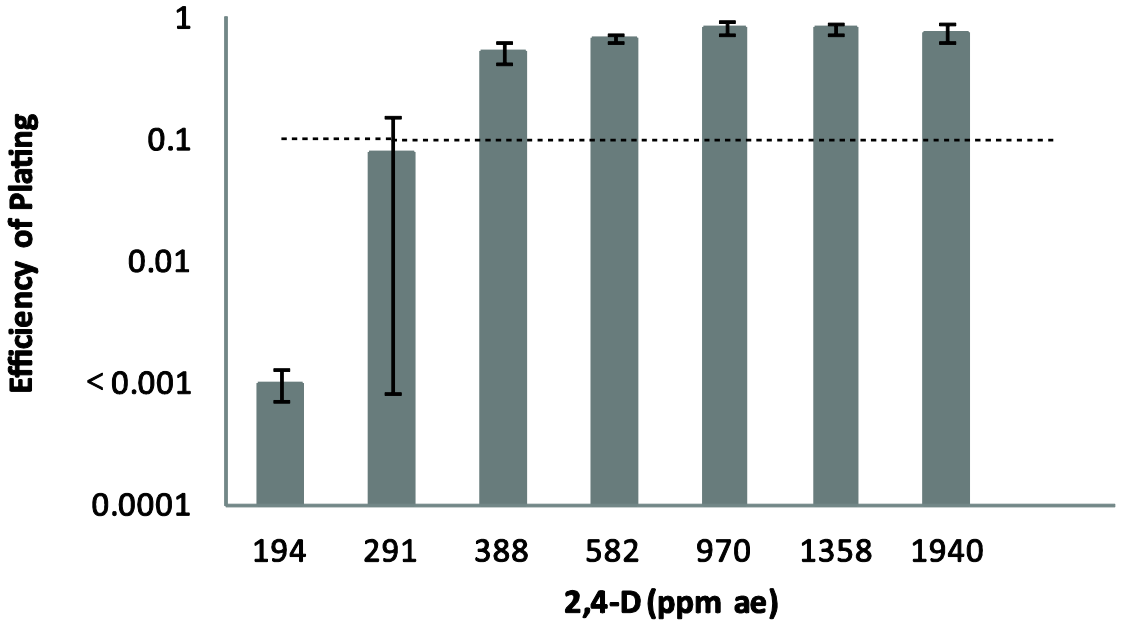

Figure 3.7. 2,4-D concentration-dependent chloramphenicol tolerance in *S. typhimurium*.
Bacteria were grown on plates with chloramphenicol (4.4 µg/ml) supplemented with various amounts of 2,4-D. Results are expressed in EOP ± SEM; the dotted line represents the differential threshold. At least one treatment is statistically significant from the other treatments (p-value = 0.0023, by Kruskal-Wallis test).

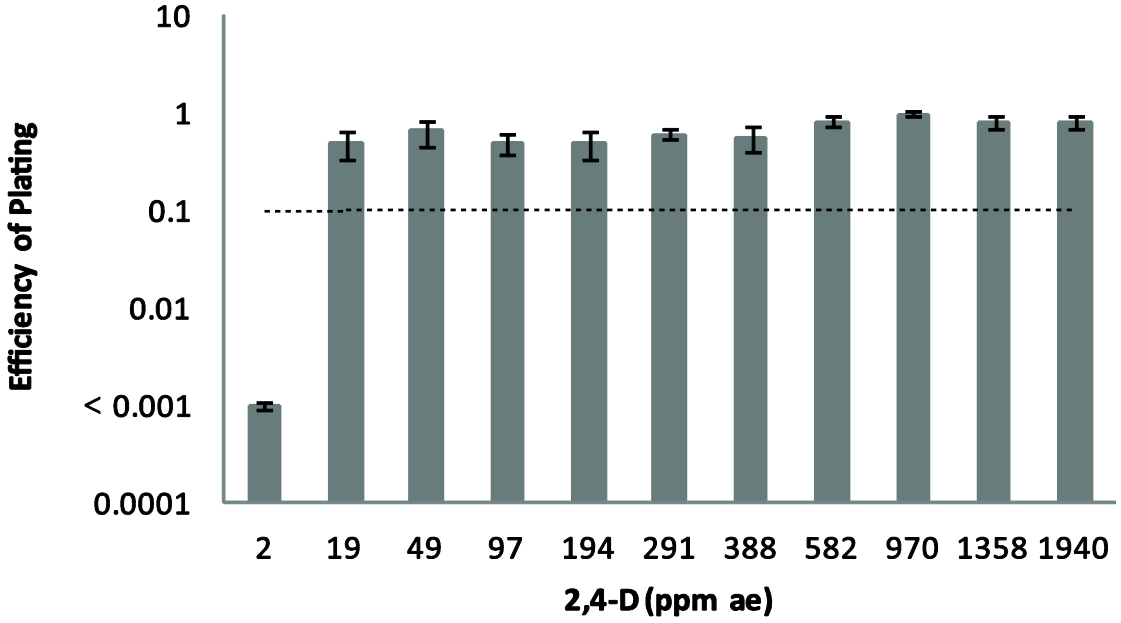

Figure 3.8. 2,4-D concentration-dependent tetracycline tolerance in *S. typhimurium*.
Bacteria were grown on plates with tetracycline (0.75 µg/ml) supplemented with various amounts of 2,4-D. Results are expressed in EOP ± SEM; the dotted line represents the differential threshold. The treatments are not statistically significant from each other at the 0.01 threshold (p-value = 0.019, by Kruskal-Wallis test).

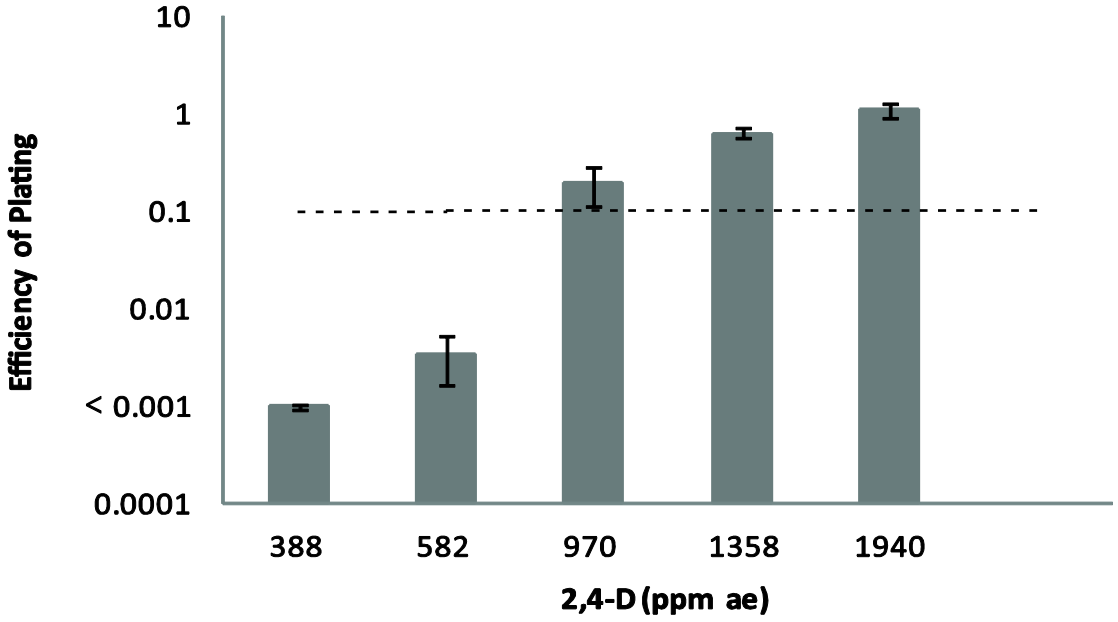

Figure 3.9. 2,4-D concentration-dependent ciprofloxacin tolerance in *S. typhimurium*.
Bacteria were grown on plates with ciprofloxacin (0.03 µg/ml) supplemented with various amounts of 2,4-D. Results are expressed in EOP ± SEM; the dotted line represents the differential threshold. At least one treatment is statistically significant from the other treatments (p-value = 0.0011, by Kruskal-Wallis test).

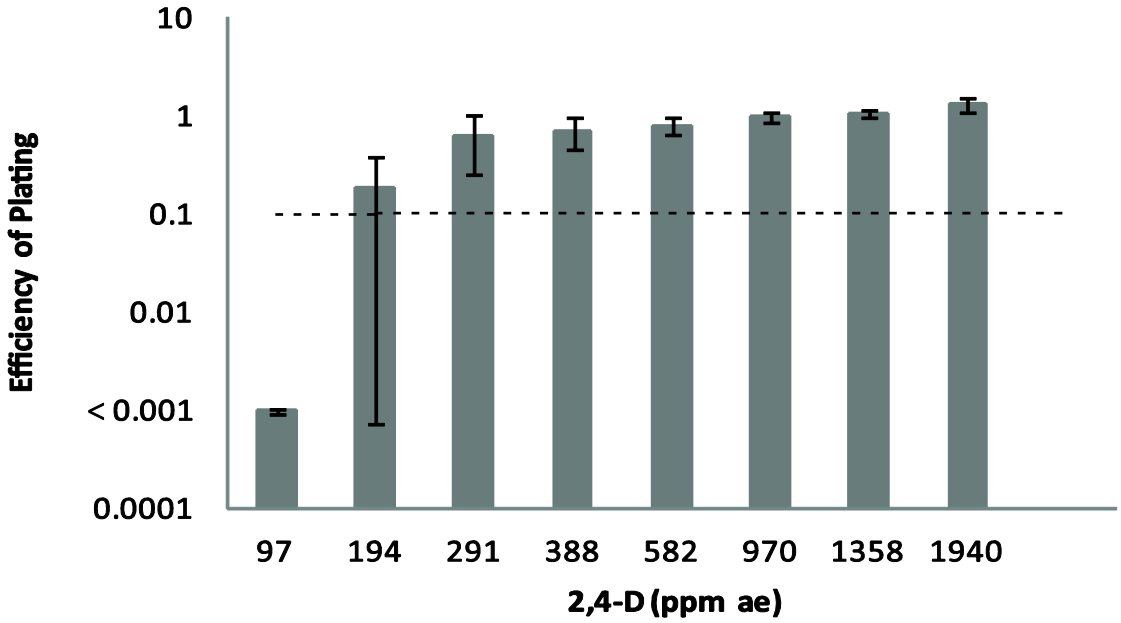

Figure 3.10. 2,4-D concentration-dependent ampicillin tolerance in *S. typhimurium*.
Bacteria were grown on plates with ampicillin (4 µg/ml) supplemented with various amounts of 2,4-D. Results are expressed in EOP ± SEM; the dotted line represents the differential threshold. The treatments are not statistically significant from each other at the 0.01 threshold (p-value = 0.013, by Kruskal-Wallis test).

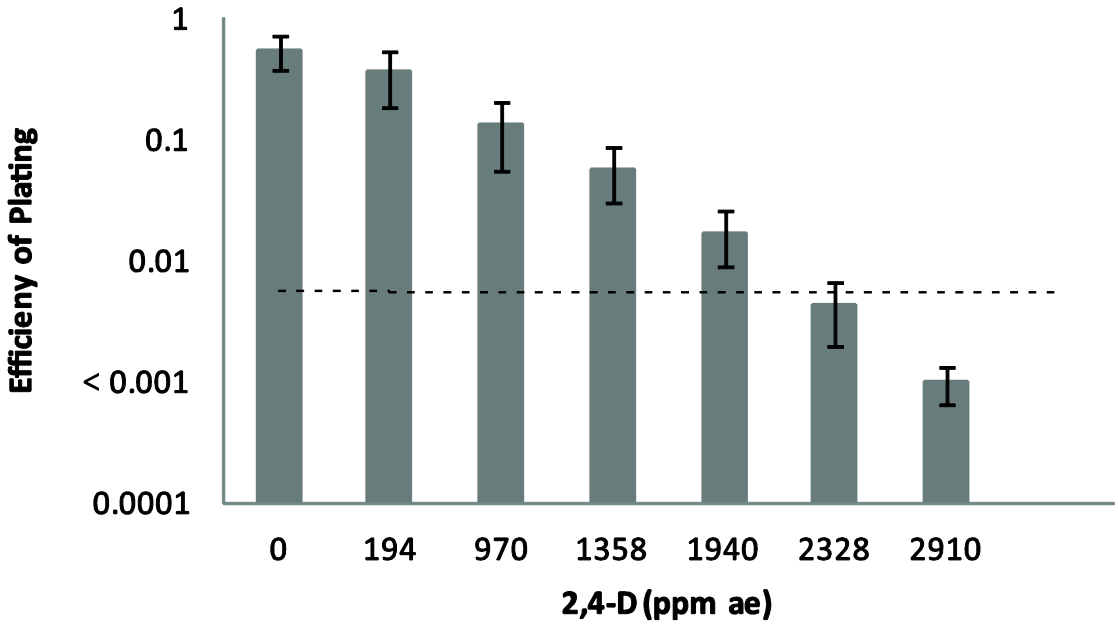

Figure 3.11. 2,4-D concentration-dependent kanamycin susceptibility in *S. typhimurium*.
Bacteria were grown on plates with kanamycin (6 µg/ml) supplemented with various amounts of 2,4-D. Results are expressed in EOP ± SEM; the dotted line represents the differential threshold. At least one treatment is statistically significant from the other treatments (p-value = 0.0038, by Kruskal-Wallis test).

3.3.3 Effect of various Roundup concentrations on antibiotic response

To study the effect of different amounts of Roundup on antibiotic response, *S. typhimurium* was incubated in the presence of antibiotics supplemented with decreasing amounts of Roundup. The antibiotic concentrations were at or above MIC for ciprofloxacin and kanamycin and below MIC for tetracycline and chloramphenicol. The same differential and conservative threshold of a 100-fold change as set for Kamba and 2,4-D, was also set for Roundup. The lowest Roundup concentration that enabled *S. typhimurium* to grow above or below the threshold was taken as the minimum inducing concentration for antibiotic tolerance or susceptibility, respectively. The effect of Roundup on ampicillin tolerance was not studied in this experiment as a previous assay (Chapter 2, section 2.3.5) showed that Roundup did not affect ampicillin tolerance on solid media.

The effect of Roundup on antibiotic response was concentration-dependent. Chloramphenicol (Fig. 3.12) and tetracycline (Fig. 3.13) *susceptibility* positively correlated with Roundup concentration while ciprofloxacin (Fig. 3.14) and kanamycin (Fig. 3.15) *tolerance* positively correlated with Roundup concentration. The minimum Roundup concentrations that induced antibiotic tolerance ranged between 621.7-704.6 ppm ae (Table 3.3). Compared to 2,4-D, the minimum induction concentrations of Roundup were within a small range. The induction concentrations of Roundup were above Kamba's induction concentrations for all antibiotics. Analysis of the data by the Kruskal-Wallis test showed that at least one treatment was significantly different from the other treatments for all antibiotics (p-value < 0.01). Based on the manufacturers guidelines, induction concentrations of Roundup were within the recommended application rates (Table 2.6). However, at the 100x EOP differential threshold the concentrations were above the MRLs set for glyphosate (Table 2.5). At a still significant but less stringent threshold of 10x EOP, the minimum inducing concentration of Roundup (435 ppm ae) for ciprofloxacin susceptibility was within the MRL range.

Table 3.3. Minimum Roundup concentrations that induced an antibiotic response

Antibiotic	Roundup (ppm ae)
Chloramphenicol[1]	621.7 ± 0.0
Tetracycline[1]	704.6 ± 82.9
Ciprofloxacin	621.7 ± 0.0
Ampicillin	N/A
Kanamycin	621.7 ± 0.0

Minimum Roundup concentrations ± SEM that cause antibiotic tolerance or susceptibility[1] in *S. typhimurium* were measured on plates and grown at 37^0C for 16-24 hours. Roundup concentrations are expressed in ppm ae.

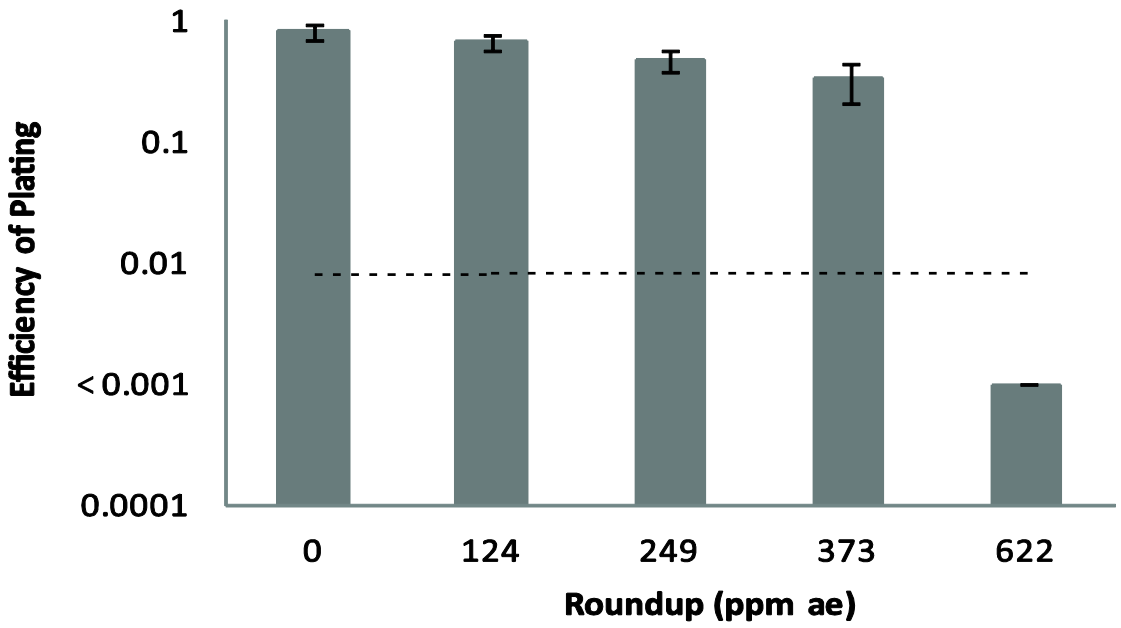

Figure 3.12. Roundup concentration-dependent chloramphenicol susceptibility in *S. typhimurium*.

Bacteria were grown on plates with chloramphenicol (2 μg/ml) supplemented with various amounts of Roundup. Results are expressed in EOP ± SEM; the dotted line represents the differential threshold. At least one treatment is statistically significant from the other treatments (p-value = 0.0016, by Kruskal-Wallis test).

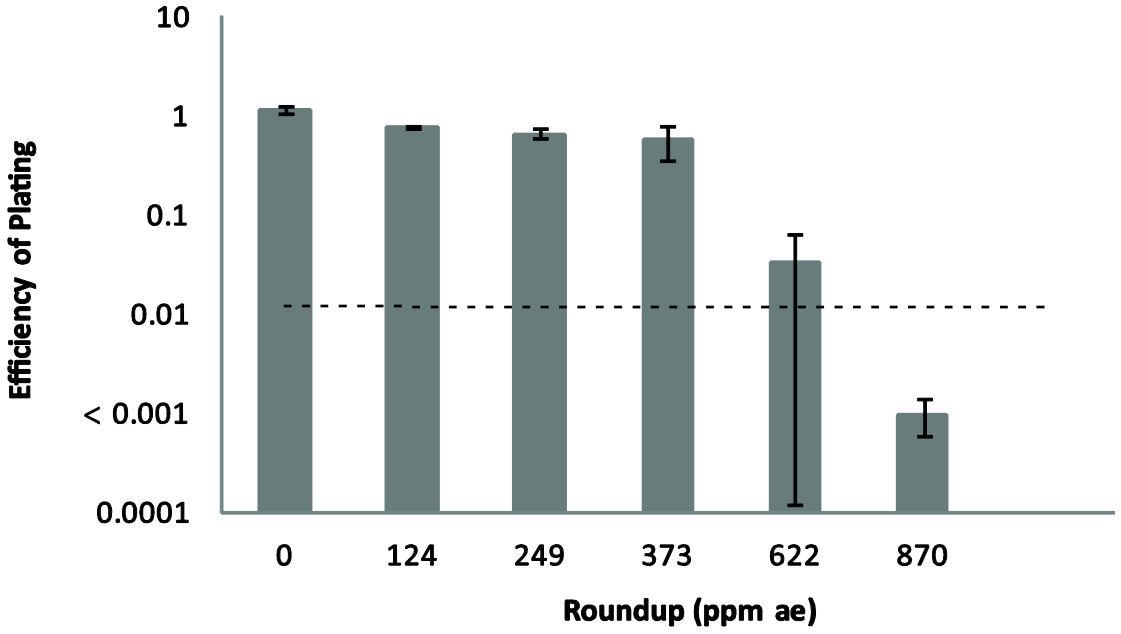

Figure 3.13. Roundup concentration-dependent tetracycline susceptibility in *S. typhimurium*.
Bacteria were grown on plates with tetracycline (0.45 µg/ml) supplemented with various amounts of Roundup. Results are expressed in EOP ± SEM; the dotted line represents the differential threshold. At least one treatment is statistically significant from the other treatments (p-value = 0.0018, by Kruskal-Wallis test).

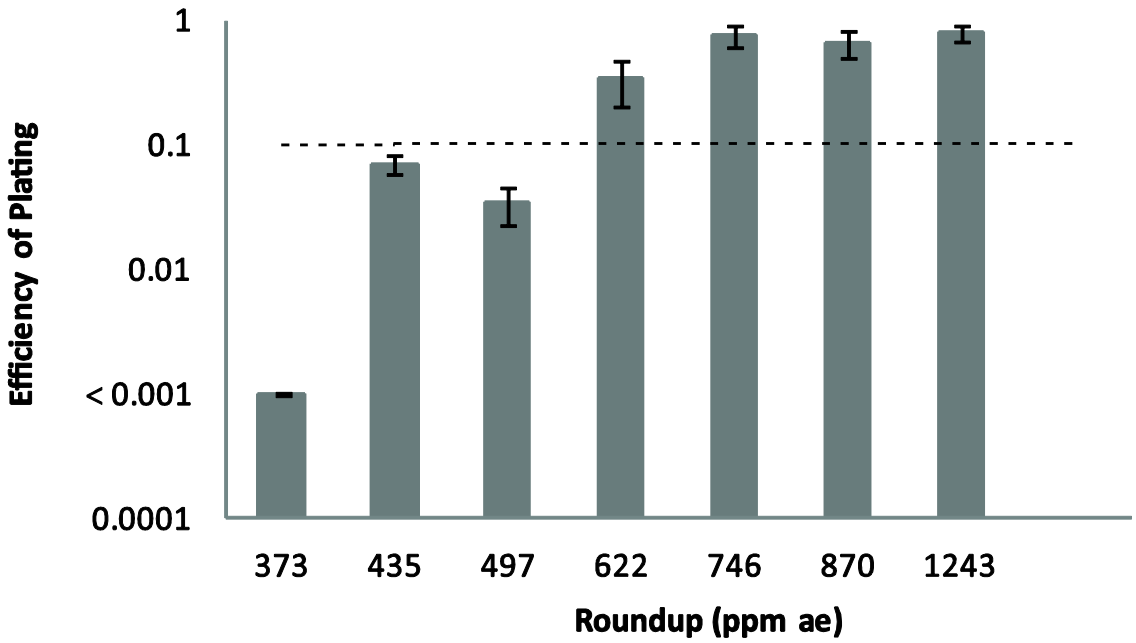

Figure 3.14. Roundup concentration-dependent ciprofloxacin tolerance in *S. typhimurium*.
Bacteria were grown on plates with ciprofloxain (0.05 µg/ml) supplemented with various amounts of Roundup. Results are expressed in EOP ± SEM; the dotted line represents the differential threshold. At least one treatment is statistically significant from the other treatments (p-value = 0.0011, by Kruskal-Wallis test).

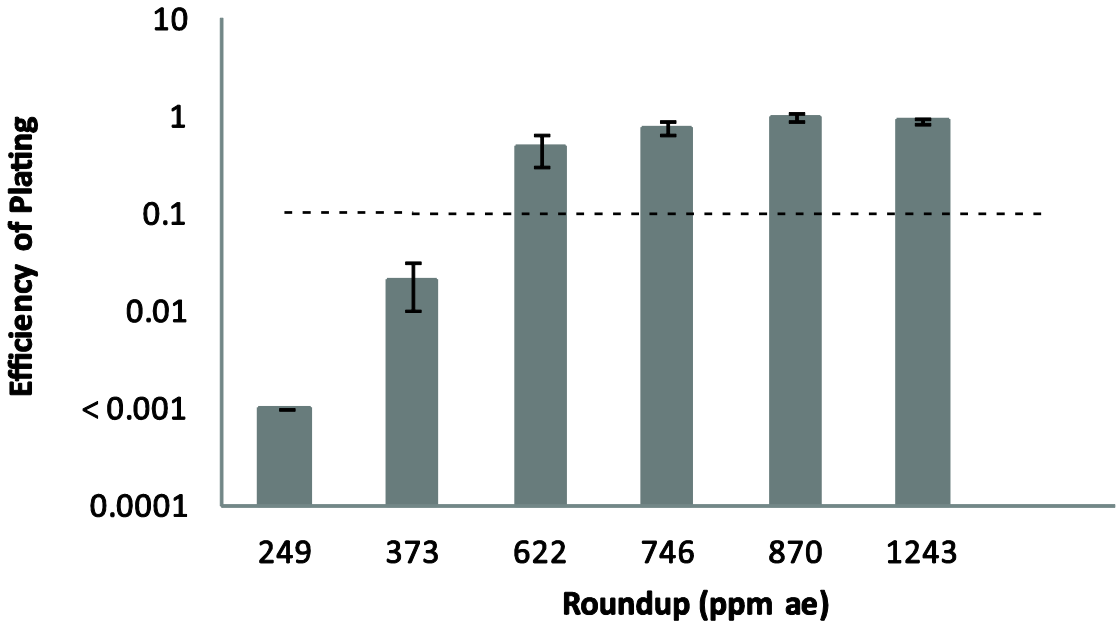

Figure 3.15. Roundup concentration-dependent kanamycin tolerance in *S. typhimurium*.
Bacteria were grown on plates with kanamycin (12 µg/ml) supplemented with various amounts of Roundup. Results are expressed in EOP ± SEM; the dotted line represents the differential threshold. At least one treatment is statistically significant from the other treatments (p-value = 0.0014, by Kruskal-Wallis test).

3.3.4 Effect of Kamba on chloramphenicol tolerance in tap water

To assess whether herbicides present in water are capable of inducing antibiotic tolerance, *S. typhimurium* was incubated in tap water under the following conditions: Kamba supplemented with 10% LB, Kamba and chloramphenicol supplemented with 10% LB, and a 'no chemical' treatment (negative control). Samples taken before and after incubation from each treatment were plated on LB plates supplemented with chloramphenicol. The difference in the number of colony forming units was calculated. For this experiment, only the herbicide Kamba and the antibiotic chloramphenicol were tested.

Initial experiments suggested that bacteria did not increase in number during incubation in water supplemented with only Kamba (results not shown), possibly due to limited nutrients in tap water. Hence, the water was supplemented with 10% LB in an effort to create conditions where the bacteria attempted to divide and thus demonstrate metabolic activity. Samples were plated on LB supplemented with chloramphenicol to determine the number of chloramphenicol-tolerant *S. typhimurium*. Although the initial concentration of bacteria used to

inoculate the media was high, there was a possibility for more growth during incubation. Yet, no detectable increase in Kamba-induced chloramphenicol tolerance was observed in water. The number of chloramphenicol-tolerant bacteria in the two chemical treatments (Kamba and Kamba plus chloramphenicol) and the no chemical treatment were not statistically different from each other at the 0.01 threshold (p-value = 0.289) (Figure 3.16).

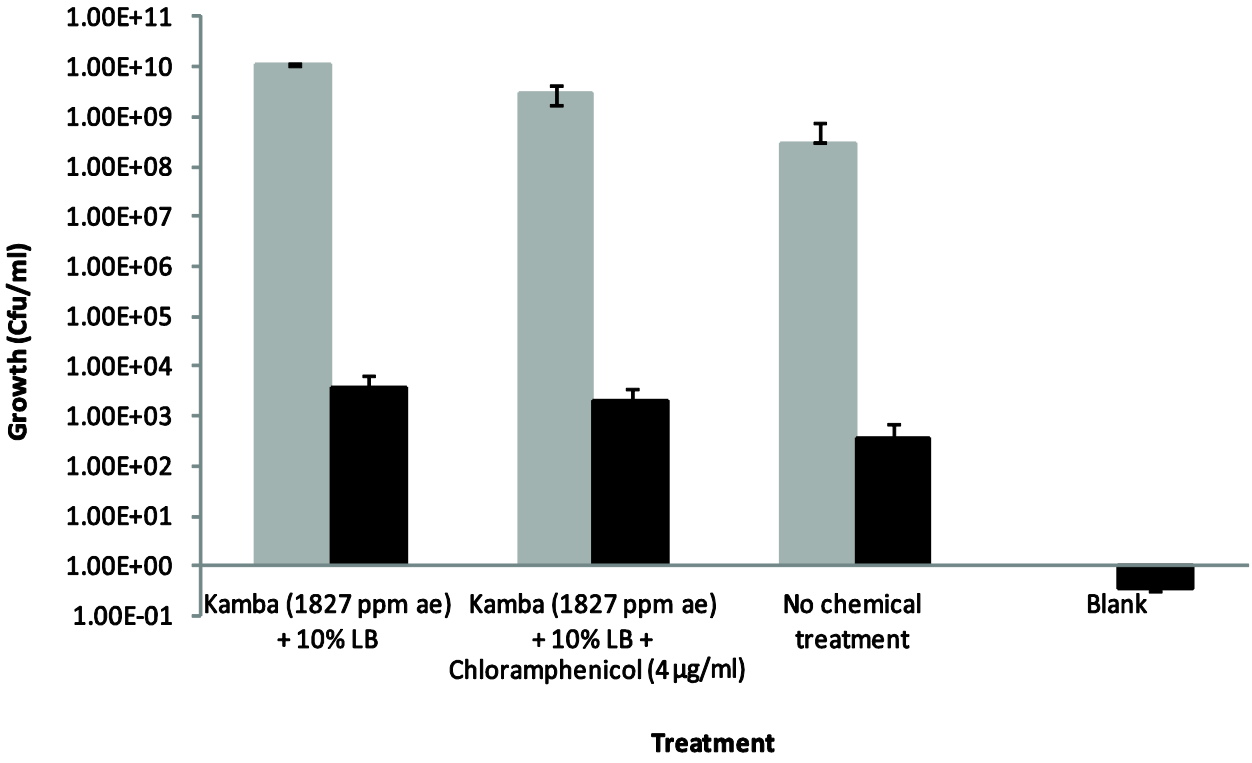

Figure 3.16. Kamba does not induce chloramphenicol tolerance in tap water.
S. typhimurium cells were incubated at 37^{0}C for 16 hours and plated on LB (Grey) and chloramphenicol (4 µg/ml) (Black). The number of *S. typhimurium* colonies that grew ± SEM are expressed in cfu/ml before treatment minus cfu/ml after treatment. The number of chloramphenicol tolerant colonies in the chemical treatments and the no chemical treatment were not statistically significant from each other at the 0.01 threashold (p-value = 0.051 for all four treatments and p-value = 0.289 for the treatments without blank, by Kruskal-Wallis test).

In the previous experiment (chapter 2), which showed a Kamba-induced effect on chloramphenicol tolerance, *S. typhimurium* was exposed to both chloramphenicol and Kamba simultaneously on plates. Here, *S. typhimurium* was first exposed to Kamba or Kamba plus chloramphenicol and then transferred to plates with only chloramphenicol (no herbicide). This may explain the absence of increased chloramphenicol tolerance. *S. typhimurium* that develop chloramphenicol tolerance may require the presence of Kamba to maintain the tolerance.

3.4 Discussion

Despite having been the focus of much attention in the scientific and wider communities in recent years, almost no research has been done on the potential effects of sub-lethal herbicide concentrations on bacteria. Results from the first part of this study showed that Kamba, 2,4-D and Roundup affected antibiotic tolerance towards chloramphenicol, tetracycline, ciprofloxacin, ampicillin (Roundup had no effect) and kanamycin. Here, the lowest concentration of Kamba, 2,4-D and Roundup that induced an antibiotic response was determined. *S. typhimurium* was grown in media that was supplemented with antibiotics and decreasing amounts of herbicides. The aim of this study was to evaluate whether antibiotic response was affected by herbicides at concentrations that are within or close to MRLs.

In addition, the effect of Kamba on chloramphenicol tolerance was studied in water to determine whether water contaminated with herbicides could induce a response. *S. typhimurium* was first incubated in water that contained Kamba and chloramphenicol, then plated on LB and chloramphenicol (above MIC) plates.

3.4.1 Lower herbicide concentrations are able to induce an antibiotic response

Results from this study revealed that an antibiotic response is elicited at lower herbicide concentrations than previously measured, some that are within MRLs. The induced antibiotic response was dependent on herbicide concentration. As the concentration of herbicide decreased the induced effect also decreased. Salicylic acid has also been shown to have a concentration-dependent effect on chloramphenicol tolerance (Rosner, 1985).

The minimum concentration of herbicide that induced an antibiotic response varied with each antibiotic. For each antibiotic the minimum induction concentration varied with each herbicide. This was expected as the different herbicides are made up of different ingredients and different concentrations of active ingredient. The chosen antibiotics were of broadly different classes as well, and thus a variance in response could be anticipated.

The minimum 2,4-D concentrations that induced chloramphenicol, tetracycline and ampicillin tolerance were within the MRL range (Table 2.5). None of the Kamba and Roundup concentrations that induce an antibiotic response were within the MRL range when measured at the 100x threshold. When the threshold was set at 10x EOP of the antibiotic-only condition, the minimum concentration of Roundup that caused ciprofloxacin tolerance was within the MRL range. Although most of the minimum inducing concentrations were above MRL, it is still possible for herbicide residues (that are within MRLs) on crops to induce some level of antibiotic response, although small.

The concentration of herbicides sprayed on crops are higher than the set MRLs and to date, there is no regulation limiting the concentrations that are sprayed. Hence, organisms that are directly exposed when the herbicide is being sprayed are likely to come into contact with higher concentrations. Humans can also come into contact with herbicides daily through a number of sources such as air, food, dust, soil and plant surfaces (Roberts & Karr, 2012). Therefore, herbicide residues may accumulate in the body and may reach concentrations that induce an antibiotic response. Glyphosate has been detected in the urine of farmers that spray this herbicide, although at low concentrations (0.2 ppm) (Acquavella *et al.*, 2004). In addition, herbicide-tolerant genetically engineered crops enable farmers to use herbicides throughout the growing season when weeds emerge (Behrens *et al.*, 2007), leading to greater accumulation of herbicides in the environment (Benbrook, 2012).

At this point it is not known whether the effect on antibiotic response is caused by the active ingredients of each herbicide or by the surfactants that are added. However, preliminary results indicate that the active ingredient alone may be sufficient for the effect (P. Gibson, B. Kurenbach and J. A. Heinemann, personal communication). Either way, this phenomenon is of concern as the herbicides are used in the environment as a commercial formulation.

The antibiotic tolerance caused by the herbicides may be due to similar pathways used by salicylic acid including an increase in efflux pumps and/or a reduction in membrane porins. The different effect on kanamycin tolerance compared to the other antibiotics tested caused by Kamba and 2,4-D may be due to the regulation of the AcrD efflux pump. This pump facilitates

the efflux of kanamycin in both *S. typhimurium* and *E. coli* (Eaves *et al.*, 2004). Eaves *et al.* (2004) suggested that *acr*D expression is either regulated by an independent mechanism to AcrAB or the number of functional AcrD pumps are limited by the number of AcrA proteins that are available to form an efflux pump (Eaves *et al.*, 2004). *S. typhimurium* grown in 2,4-D and Kamba showed increased *susceptibility* to kanamycin but increased *tolerance* to other antibiotics tested. This suggests that these herbicides increase the expression of AcrAB, without raising AcrD-TolC levels. On the other hand, the antibiotic response caused by Roundup, suggests that it increases AcrD (causing kanamycin tolerance).

Similar to the results presented here, Rosner (1985) reported a concentration-dependent increase in chloramphenicol tolerance when *E. coli* was exposed to salicylic acid (Rosner, 1985). This suggests that the antibiotic response observed here may be caused by efflux pumps. Low herbicide concentrations may allow an increase in efflux pumps in a few bacterial cells while high herbicide concentrations are necessary to induce tolerance in all the cells of a culture. Further research is necessary to determine the particular genes that are up-regulated or down-regulated in the presence of herbicides.

Future experiments to determine the up-regulation of particular genes in bacteria exposed to herbicide can be detected through Reverse transcription polymerase chain reaction (RT-PCR). Quantitative RT-PCR is a powerful tool for detecting RNA levels (Freeman *et al.*, 1999). Briefly, RNA is first isolated from cultures and then using a reverse transcriptase and primers, a single-strand complementary DNA (cDNA) is produced. The cDNA is then amplified using quantitative PCR (Freeman *et al.*, 1999).

The expression levels of *acrA*, *acrB, acrD*, *soxS* and *marA* should be determined by quantitative RT-PCR for cultures exposed to herbicides. Both *soxS* and *marA* are known to be global activators of the *acrRAB* locus in *S. typhimurium* (Eaves *et al.*, 2004) and activation of these genes have been shown to increase tolerance towards antibiotics such as chloramphenicol (Zheng *et al.*, 2009). The expression of the efflux pumps should also be determined as they may be activated by other pathways such as *ramA* (Cohen *et al.*, 1993; Zheng *et al.*, 2009).

To do this, *S. typhimurium* should first be grown as a day culture in the following conditions: LB, LB supplemented with antibiotic, LB supplemented with antibiotic and herbicide, LB supplemented with herbicide and a blank without culture. Extracted RNA should be converted to cDNA and levels quantified by real-time quantitative PCR (qPCR). There are two methods that can be used in qPCR, absolute and relative quantification. For the purposes of this study, relative quantification can be used. This technique compares the fold difference in gene expression of the target genes with reference genes in the same bacteria in order to correct for differences in the quality of cDNA (Taylor *et al.*, 2010). Serial dilutions of specific housekeeping genes or the genes from no herbicide/antibiotic treatment can be used to generate a standard curve. This can be used to compare the fold increase or decrease in gene expression between cells that have been treated with herbicide and/or antibiotic.

According to the results gathered here, the up-regulation of *marA, soxS*, *acrA* and *acrB* and down-regulation of *acrD* can be expected in cells exposed to Kamba and 2,4-D. On the other hand, cultures grown in the presence of Roundup, an increase in *acrD* and *arcA* expression can be expected. If there is an increase in *acrA, acrB* and *acrD* but no increase in *marA* or *soxS* expression, it would suggest that the herbicides activate the efflux pumps through other pathways. The same experiment could be extended to determine expression of porin genes such as *micF*.

3.4.2 Kamba does not affect chloramphenicol tolerance in tap water

In this study, Kamba did not affect chloramphenicol tolerance in *S. typhimurium* that were incubated in tap water. *S. typhimurium* and other bacteria are known to contaminate water sources (Jacobsen & Bech, 2012). In addition, herbicides and antibiotics can also contaminate water through runoff (Davis *et al.*, 2006; Shipitalo *et al.*, 2008), creating an environment where bacteria are exposed to herbicides and antibiotics. Contaminated water may contain other nutrients, especially around farms, enabling bacteria to survive and grow, allowing herbicides to potentially affect antibiotic response.

Cultures that were first exposed to Kamba did not have higher chloramphenicol tolerance compared to the no treatment culture. The Kamba concentration used during incubation

(1827 ppm ae) was above the minimum inducing concentration (274 ppm ae) determined in the previous study. This may suggest that *S. typhimurium* has to be exposed to Kamba and chloramphenicol simultaneously to cause chloramphenicol tolerance. Hence, cells were incubated in water supplemented with Kamba and chloramphenicol (above MIC). However, cultures from this treatment also did not show increased tolerance.

In previous experiments that showed increased chloramphenicol tolerance due to Kamba (Chapter 2), *S. typhimurium* were plated on media containing Kamba and chloramphenicol. Here, although initially exposed to Kamba and chloramphenicol, *S. typhimurium* was plated on media containing chloramphenicol-only, suggesting that bacteria may require the presence of both chemicals in order to display antibiotic tolerance. Experiments that include plating cultures (that have been incubated in water supplemented with Kamba) on plates with chloramphenicol and Kamba would be worthy of future work.

Further testing is also necessary because the response toward other antibiotics may be different. The response may also vary with each strain. For example, Kamba has no effect on ampicillin tolerance in *E. coli* (B. Kurenbach and J.A. Heinemann, personal communication) while it increases tolerance in *S. typhimurium*. Similarly, Rosner (1985) found that induction of chloramphenicol tolerance varied with strains as well as with different chemical inducers (Rosner, 1985).

Chapter Four

4. Maintaining Herbicide-induced Antibiotic Tolerance and Determining the Effect of Herbicide Combinations on Antibiotic Tolerance

4.1 Introduction

The previous chapters provided the first evidence of an herbicide-induced antibiotic response in *Salmonella enterica* serovar *Typhimurium* (Chapter 2). The herbicides - Kamba, 2,4-D and Roundup induced a response towards chloramphenicol, tetracycline, ciprofloxacin, ampicillin (not affected by Roundup) and kanamycin at herbicide concentrations below the recommended application rates and some below Maximum Residue Limits (MRLs) (Chapter 3).

Some gene regulatory systems, such as activation of the *lac* operon, have been shown to require a high concentration of a substance to induce a phenotype. Once induced, the phenotype can be maintained at lower concentrations (Novick & Weiner, 1957; Vilar *et al.*, 2003). Bacteria that come into contact with herbicides at application rate concentrations may respond to this in many ways, including displaying a change in antibiotic susceptibility. However, bacteria may not receive constant exposures to high concentrations of herbicide; instead they may be exposed to lower herbicide concentrations that may be present in waterways or soil.

This raised the question of whether the antibiotic response induced by herbicides can be maintained at lower concentrations or even in the absence of herbicide. To explore this possibility, we chose to use Kamba and *S. typhimurium.* The bacteria were pre-induced by Kamba with or without antibiotic, and then transferred to media with either antibiotic or antibiotic with Kamba (below inducing concentrations). This assay was performed on plates for chloramphenicol and in 96-well plates for chloramphenicol, tetracycline, ampicillin and ciprofloxacin. To determine if antibiotic tolerance was maintained, the titre from plate counts

and the optical densities of cultures in 96 well plates were recorded. Furthermore, colonies that were tolerant to chloramphenicol were serially re-streaked for 5 days on LB and chloramphenicol plates to determine if antibiotic tolerance is returned to its normal state in herbicide free media.

In addition, the effect of herbicide combinations in inducing an antibiotic response was examined. Previous work demonstrated responses in opposite directions depending on which herbicide was used. For example, Roundup increased *tolerance* to kanamycin while Kamba increased *sensitivity* to kanamycin. To determine if these effects were additive, *S. typhimurium* was exposed to either chloramphenicol or kanamycin and two herbicides simultaneously: Kamba plus Roundup or Kamba plus 2,4-D. The antibiotic concentrations ranged from below Minimum Inhibitory Concentration (MIC) to above MIC. The changes in antibiotic MIC were determined by examining cultures visually for cloudiness indicating growth. Other antibiotics were not included in this assay.

With the development of herbicide-tolerant weeds, farmers are being forced to increase the number of herbicide applications (Green, 2014) and are using mixtures of herbicides such as Banvine™ (dicamba and 2,4-D) (Gawn *et al.*, 2013). New commercial formulations of herbicides are also being developed with dual active ingredients, like Enlist Duo®™ which contains glyphosate and 2,4-D (Ford *et al.*, 2014). Furthermore, crops are being engineered to have tolerances to multiple herbicides to enable use of dual herbicide mixtures (Ford *et al.*, 2014). The majority of studies on the effects of herbicides focus mainly on individual herbicides (Bukowska, 2006; Gonzalez *et al.*, 2006; Clair *et al.*, 2012) and there is no previous research assessing the effects of herbicide combinations on bacteria in relation to antibiotic response.

4.2 Methods

4.2.1 Bacterial strains and growth conditions

Bacterial cultures were grown and maintained as described in Chapter 2 (section 2.2.1). All assays were done with *Salmonella enterica* serovar *Typhimurium* strain SL3770 (*rfa*$^{+}$) (MacLachlan & Sanderson, 1985), and the three commercial herbicides: Kamba, 2,4-D and Roundup.

4.2.2 Maintaining antibiotic tolerance induced by herbicides

4.2.2.1 Maintaining Kamba induced chloramphenicol tolerance on solid media

An assay was done to determine whether chloramphenicol tolerance induced by Kamba can be maintained either at low Kamba concentrations or in its absence. *S. typhimurium* was grown in Luria Broth (LB) at 37^{0}C until the OD_{600} reached 1. Then 100µl aliquots were incubated in the following liquid conditions: LB, LB supplemented with chloramphenicol (6 µg/ml), LB supplemented with chloramphenicol (6 µg/ml) plus Kamba (731 ppm ae) and LB supplemented with Kamba (731 ppm ae and 7310 ppm ae) (Figure 4.1 – A). LB without culture was included as a blank. The total volume of each condition was 10 ml. The cultures were incubated at 37^{0}C in a shaking incubator for 16-48 hours.

Following incubation, bacterial cells from each condition (1ml) were harvested by centrifugation at 6613 x *g* for 90 seconds. The supernatant was discarded and the cell pellet was re-suspended in LB. Aliquots from each condition were serially diluted 10-fold in LB. The three dilutions were immediately plated in replicate on the following plate treatments: LB, LB supplemented with chloramphenicol (6 µg/ml), LB supplemented with chloramphenicol (6 µg/ml) plus increasing amounts of Kamba (18 ppm ae, 183 ppm ae, 731 ppm ae). During plating, the diluted cultures were stored on ice to prevent bacterial growth. The plates were incubated at 37^{0}C for 16-48 hours. The titres for each treatment were determined and the Efficiency Of Plating (EOP) (as described in Chapter 2, section 2.2.3.1) was calculated relative to the titre (cfu/ml) of the LB control within each condition.

A sample of colonies from each condition was then re-streaked on LB, chloramphenicol (6 µg/ml) and chloramphenicol (6 µg/ml) plus Kamba (731 ppm ae) plates (Figure 4.1-B). The plates were incubated at 37^0C for 16-20 hours. Colonies that showed chloramphenicol tolerance were serially re-streaked onto LB for 5 days. Each day, colonies that grew on LB were re-streaked on chloramphenicol (6 µg/ml) to determine whether colonies can maintain chloramphenicol tolerance.

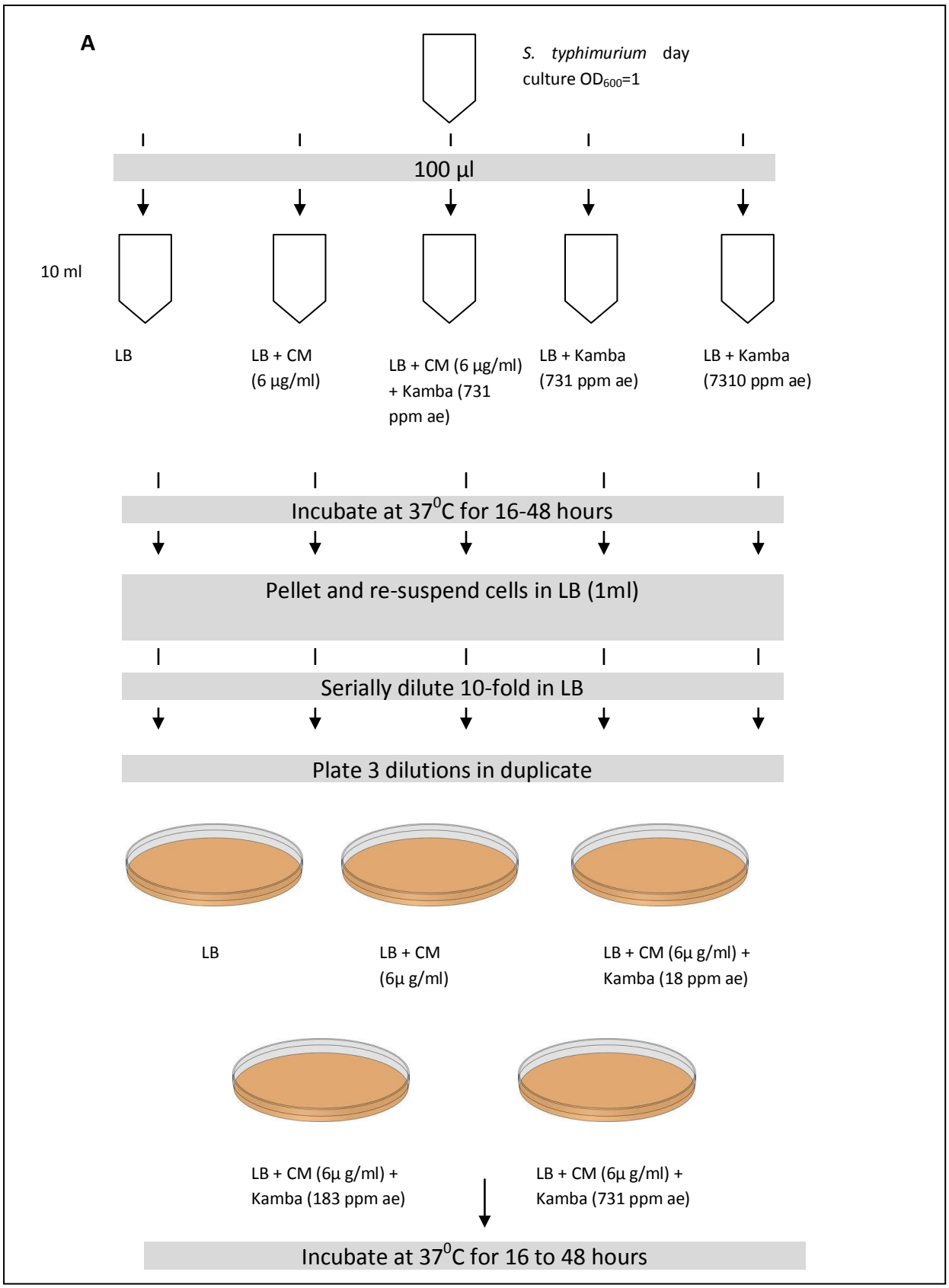

Figure 4.1. Schematic diagram of the maintenance experiment.

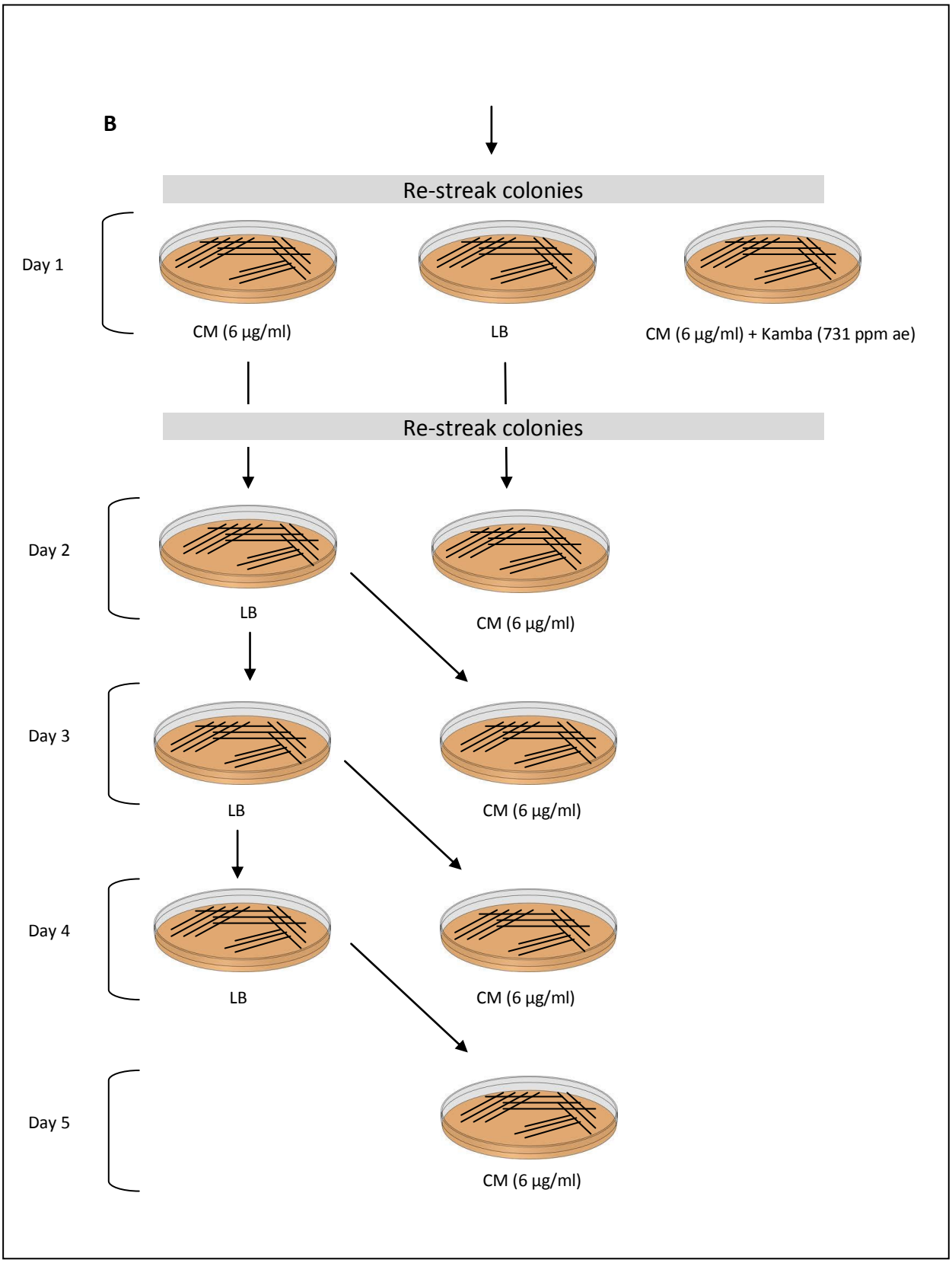

Figure 4.1. Schematic diagram of the maintenance experiment

4.2.2.2 Maintaining herbicide-induced antibiotic tolerance in liquid media

Maintenance of antibiotic tolerance induced by herbicides was also determined in liquid media. *S. typhimurium* was grown in liquid LB at 37^0C until the culture reached an OD_{600} of 1. Similar to the method used above (4.2.2.1) cultures were incubated in the following conditions: LB, LB supplemented with antibiotic (above MIC) and LB supplemented with antibiotic (above MIC) along with increasing amounts of Kamba (731 ppm ae, 7310 ppm ae and 10965 ppm ae). The total volume of each condition was 10 ml. Cultures were incubated at 37^0C for 16-48 hours in a shaking incubator.

Following incubation, bacterial cells from each condition (1ml) were harvested by centrifugation at 6613 x *g* for 90 seconds. The supernatant was discarded and the pellet was re-suspended in LB (1ml). In a 96 well plate, liquid media consisting of no chemical treatment, antibiotic (above MIC) and antibiotic (above MIC) plus increasing concentrations of Kamba (from 18 ppm ae to 731 ppm ae) were prepared in duplicate for each induction condition. Immediately after the cells were re-suspended in LB, aliquots from each culture condition were added to the wells. The total volume of each well was 200 µl. A blank was included as a control. The cultures were incubated at 37^0C for 34 hours 35 minutes in a FLUOstar-OPTIMA plate reader which measured the optical density of the cultures at 595 nm at regular intervals. The settings of the plate reader are given below.

FLUOstar OPTIMA plate reader settings

Positioning delay = 1
Number of kinetic windows = 1
Number of cycles = 250
Measurement start time = 0.0 seconds
Number of flashes per well and cycle = 10
Cycle time = 500 seconds
Number of multichromatics = 1
Excitation filter = A-595
Emmision filter = empty
Orbital averaging = ON
Diameter = 2 nanometers
Total cycle time = 34 hours 35 minutes

4.2.3 Determining the effect of herbicide combinations on antibiotic response

The effect of herbicide combinations on antibiotic response was determined in liquid media. *S. typhimurium* was grown in liquid LB at 37^0C until the culture reached an OD_{600} of 1. In 24 well plates, LB with antibiotic (ranging from below MIC to above MIC) was added to each well, across rows. The two antibiotics, chloramphenicol and kanamycin were tested. To each row the following treatments were added: no herbicide, Kamba (1827 ppm ae), 2,4-D (1940 ppm ae), Roundup (1243 ppm ae), Kamba (1827 ppm ae) plus 2,4-D (1940 ppm ae) and Kamba (1827 ppm ae) plus Roundup (1243 ppm ae). An aliquot (10 µl) of the culture was added to each well bringing the total volume to 1 ml. Controls included no chemical treatment and a blank. The plates were incubated in a Thermolyne-ROSI 1000 orbital shaking incubator at 185 rpm and at 37^0C for 16-20 hours. The cultures were examined visually for cloudiness, indicating growth.

4.2.4 Statistical analysis

As described in Chapter 3, section 3.2.4, the Kruskal-Wallis test, was used for the maintenance assay in both liquid and solid media because the data was not normally distributed. To analyze the data, the statistical difference between the pre-treatments was tested with the null hypothesis for each incubation condition. For the assay on solid media, the EOPs of each plate condition were compared with each pre-treatment. For example, the EOPs of the chloramphenicol plates were compared between the pre-treatments: chloramphenicol (6µg/ml), chloramphenicol (6 µg/ml) plus Kamba (731 ppm ae), Kamba (731 ppm ae), Kamba (7310 ppm ae) and the no pre-treatment.

For the assay done in liquid media, the OD_{600} of each well treatment (no chemical treatment, antibiotic, antibiotic plus Kamba (18 ppm ae to 731 ppm ae)) was compared between the pre-treatments (LB, LB plus antibiotic, LB plus antibiotic with Kamba (731 ppm ae, 7310 ppm ae and 10965 ppm ae)), for each antibiotic, at the start and finish of incubation. The p-value threshold was set at < 0.01, meaning that the likelihood of the observed result occurred by chance alone is less than 1%.

4.3 Results

4.3.1 Chloramphenicol tolerance induced by Kamba was maintained at low and nil Kamba concentrations

The maintenance of chloramphenicol tolerance induced by Kamba in *S. typhimurium* was studied. *S. typhimurium* was first pre-treated by exposure to chloramphenicol (above MIC), chloramphenicol and Kamba (731 ppm ae), and two concentrations of Kamba (731 ppm ae and 7310 ppm ae). The two Kamba concentrations used in this assay were both above inducing concentrations (Chapter 3, section 3.3.1). Initial experiments suggested that 731 ppm ae Kamba was not sufficient to maintain chloramphenicol tolerance; hence the higher Kamba concentration of 7310 ppm ae (below MIC) was also tested. Following pre-treatment, the bacteria were harvested from liquid cultures and transferred to solid media composed of: LB with and without chloramphenicol, and chloramphenicol with Kamba. The Kamba concentrations ranged from below (18 ppm ae) to above minimum inducing concentrations (731 ppm ae). A below inducing concentration was included to determine if tolerance can be maintained at lower levels once tolerance was induced. The number of colonies that grew following each treatment was counted and converted to an EOP. Due to variation in the number of bacterial cells that grew in each condition during pre-treatment, the EOP was calculated by dividing the cfu/ml of each plate by the cfu/ml of the LB condition within each pre-treatment.

S. typhimurium that was pre-treated with chloramphenicol and Kamba maintained chloramphenicol tolerance at lower amounts of Kamba and even in its absence (Fig. 4.2). It is interesting to note that two sizes of colonies grew, a small and a big size, on plates with chloramphenicol and 731 ppm ae Kamba. The total number of colonies (small and big) were used in calculating EOP.

When *S. typhimurium* was pre-treated with Kamba-only, chloramphenicol tolerance was not maintained at both the concentrations tested. The cultures from these pre-treatments had chloramphenicol tolerance when a minimum of 183 ppm ae Kamba was present in the media.

When *S. typhimurium* was pre-treated with chloramphenicol-only, a few cells maintained tolerance, but not to the same level as cells pre-treated with chloramphenicol and Kamba.

Maintenance of chloramphenicol tolerance in the absence of Kamba was higher when *S. typhimurium* was pre-treated with chloramphenicol and Kamba than when pre-treated with Kamba-only, chloramphenicol-only and no-treatment. However, the result was only marginally significant at the p-value < 0.01 threshold (p-value = 0.016). The number of *S. typhimurium* that were tolerant to chloramphenicol at below inducing (18 ppm ae) concentrations was higher when cells were pre-treated with chloramphenicol and Kamba compared to the other treatments. However, the result was only marginally significant at the p-value < 0.01 threshold (p-value = 0.027). The results suggest that Kamba alone is not sufficient for inducing antibiotic tolerance. The effect requires the antibiotic also.

Colonies that were tolerant to chloramphenicol were serially re-streaked on LB for 5 days and were re-tested for maintenance of chloramphenicol tolerance each day. Of the cultures pre-treated with chloramphenicol, colonies that were subsequently plated on chloramphenicol or 731ppm ae Kamba plus chloramphenicol, remained tolerant. Cultures pre-treated with chloramphenicol plus Kamba remained tolerant regardless of the plate condition. That is, once induced by simultaneous exposure to both the herbicide and antibiotic, the effect was maintained independently of either chemical agent. However, only the big sized colonies from the Kamba (731 ppm ae) plus chloramphenicol plates maintained tolerance for 5 days. This suggests that incubation of *S. typhimurium* in Kamba and chloramphenicol may give rise to two populations, one that is tolerant only when Kamba is present and one that does not require the presence of Kamba.

Colonies which maintained tolerance did not come into contact with Kamba for at least 5 days. The colonies were serially transferred to LB plates for 5 days and then re-tested for tolerance. This suggests that once activated, herbicide-induced tolerance remains activated, or that the presence of another antimicrobial (chloramphenicol) prevents the ‘switch’ back to susceptibility.

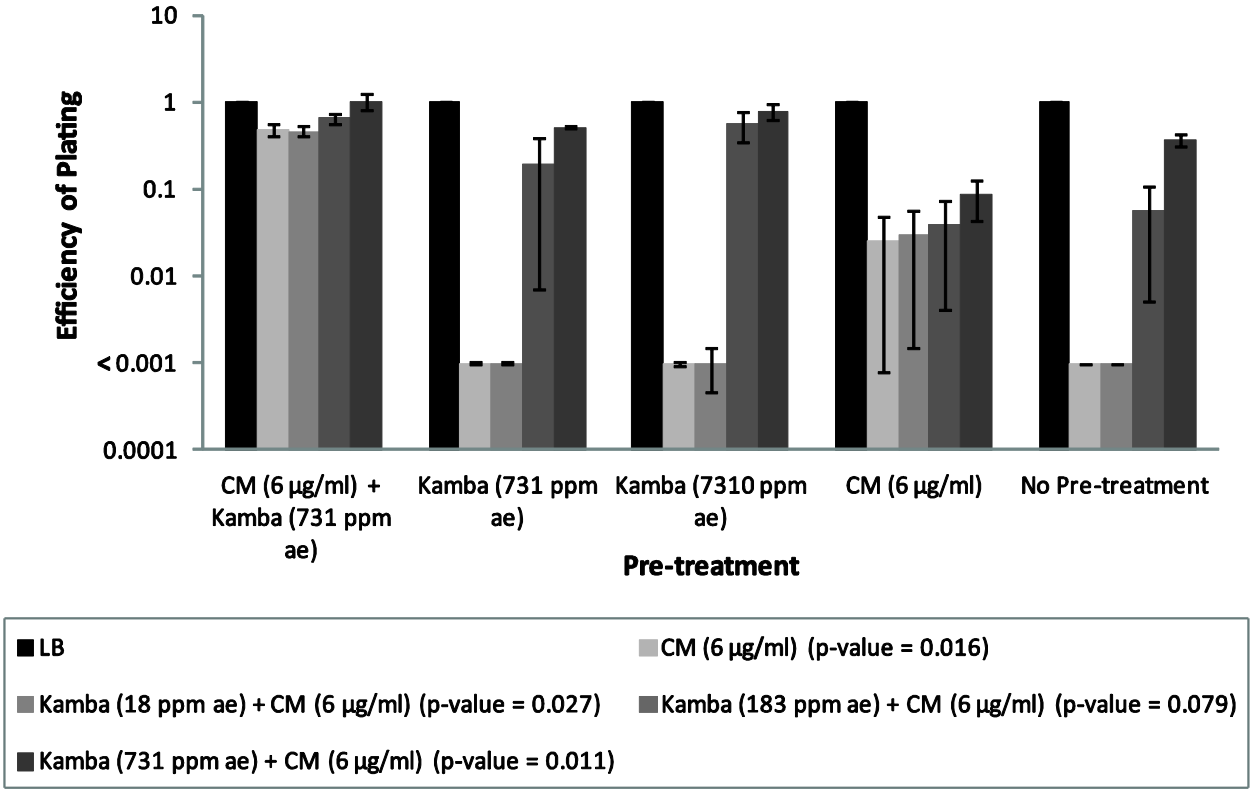

Figure 4.2. Maintenance of chloramphenicol tolerance in *S. typhimurium* on solid media.
Bacteria were pre-treated with 5 different conditions and then plated on LB, chloramphenicol (CM) (6 µg/ml) and CM (6 µg/ml) plus Kamba (18, 183 and 731 ppm ae). Results are expressed in EOP ± SEM (n=3) and the p-values were calculated using the Kruskal-Wallis test. None of the EOPs of the plate conditions were statistically different between pre-treatments at the 0.01 threshold.

4.3.2 Maintenance of antibiotic tolerance induced by Kamba in liquid media

The maintenance of antibiotic tolerance was also tested in liquid media. *S. typhimurium* was first pre-treated (16-48 hours) in the following conditions: antibiotic (above MIC) plus Kamba (731 ppm ae), Kamba-only (731, 7310 and 10965 ppm ae), antibiotic-only and no chemical treatment. Then the bacterial cells were harvested from media and incubated in a 96-well plate. The wells were made up of the following conditions: LB, antibiotic (above MIC) and antibiotic (above MIC) with Kamba (1.8 ppm ae to 731 ppm ae). The growth of bacteria was measured by the plate reader as optical density over 34 hours.

4.3.2.1 Chloramphenicol

Similar to the results of the maintenance assay on solid media, *S. typhimurium* pre-treated with chloramphenicol and Kamba (731 ppm ae) maintained tolerance in the presence of Kamba, below inducing concentrations, and in the absence of Kamba (Fig. 4.3 A). When cultures were pre-treated with 731 ppm ae Kamba, tolerance was not maintained either in the absence of Kamba or with ongoing Kamba exposures below 731 ppm ae (Fig. 4.3 B).

Pre-treatment with 7310 ppm ae (Fig. 4.4 A) and 10965 ppm ae (Fig. 4.4 B) Kamba provided only partial tolerance (optical density remained low) in the absence of Kamba and with Kamba below induction concentrations. This suggests that treatment with high concentrations of Kamba may affect chloramphenicol tolerance although the effect is small. Cultures that were not pre-treated (Fig. 4.5 B) or pre-treated with chloramphenicol-only (Fig. 4.5 A) did not show maintenance of tolerance, consistent with the results from solid media.

The final optical densities for each incubation condition were compared between pre-treatments using the Kruskal-Wallis test. For example, the optical densities of the chloramphenicol plus 1.8 ppm ae Kamba condition were compared between all six pre-treatments. None of the conditions were significantly different between the pre-treatments at the 0.01 p-value threshold (Table 4.1). However, compared to the p-values at the beginning of incubation (Time = 0) the p-values after incubation decreased for most conditions. For the chloramphenicol plus 1.8 ppm ae Kamba condition, the p-value reduced from 0.344 to 0.033, indicating that it may be marginally significant and suggests that there may be a difference in at least one of the pre-treatments after incubation.

Table 4.1. p-values for the maintenance of chloramphenicol tolerance induced by Kamba

Condition	p-value	
	Time = 0 hours	Time = 34 hours 35 minutes
Chloramphenicol-only	0.126	0.075
No chemical treatment	0.163	0.179
Chloramphenicol + Kamba (1.8 ppm ae)	0.344	0.033
Chloramphenicol + Kamba (18 ppm ae)	0.413	0.022
Chloramphenicol + Kamba (91 ppm ae)	0.238	0.032
Chloramphenicol + Kamba (183 ppm ae)	0.076	0.117
Chloramphenicol + Kamba (731 ppm ae)	0.745	0.161

p-values before (time = 0) or after incubation (time = 34 hours 35 minutes), by the Kruskal-Wallis test.

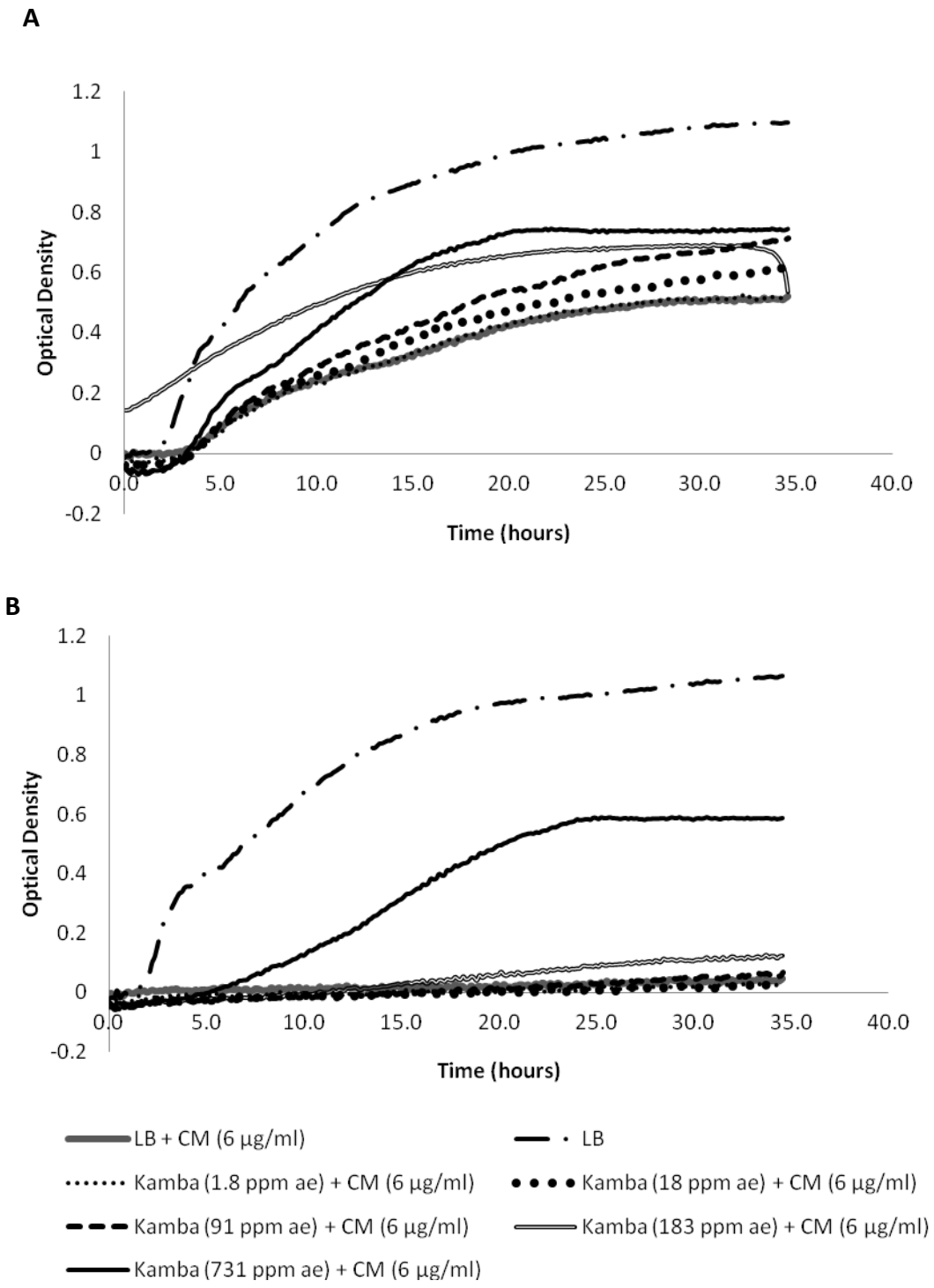

Figure 4.3. Effect on chloramphenicol tolerance in *S. typhimurium* pre-treated with chloramphenicol plus Kamba and Kamba-only.

Bacteria were pre-treated with chloramphenicol (6 µg/ml) plus Kamba (731 ppm ae) **(A)** and Kamba-only (731 ppm ae) **(B)**. Cells were then incubated in LB, chloramphenicol (CM) (6 µg/ml) and CM (6 µg/ml) plus Kamba (1.8, 18, 91, 183 and 731 ppm ae). Results are expressed as optical density measured over time, mean of three independent experiments.

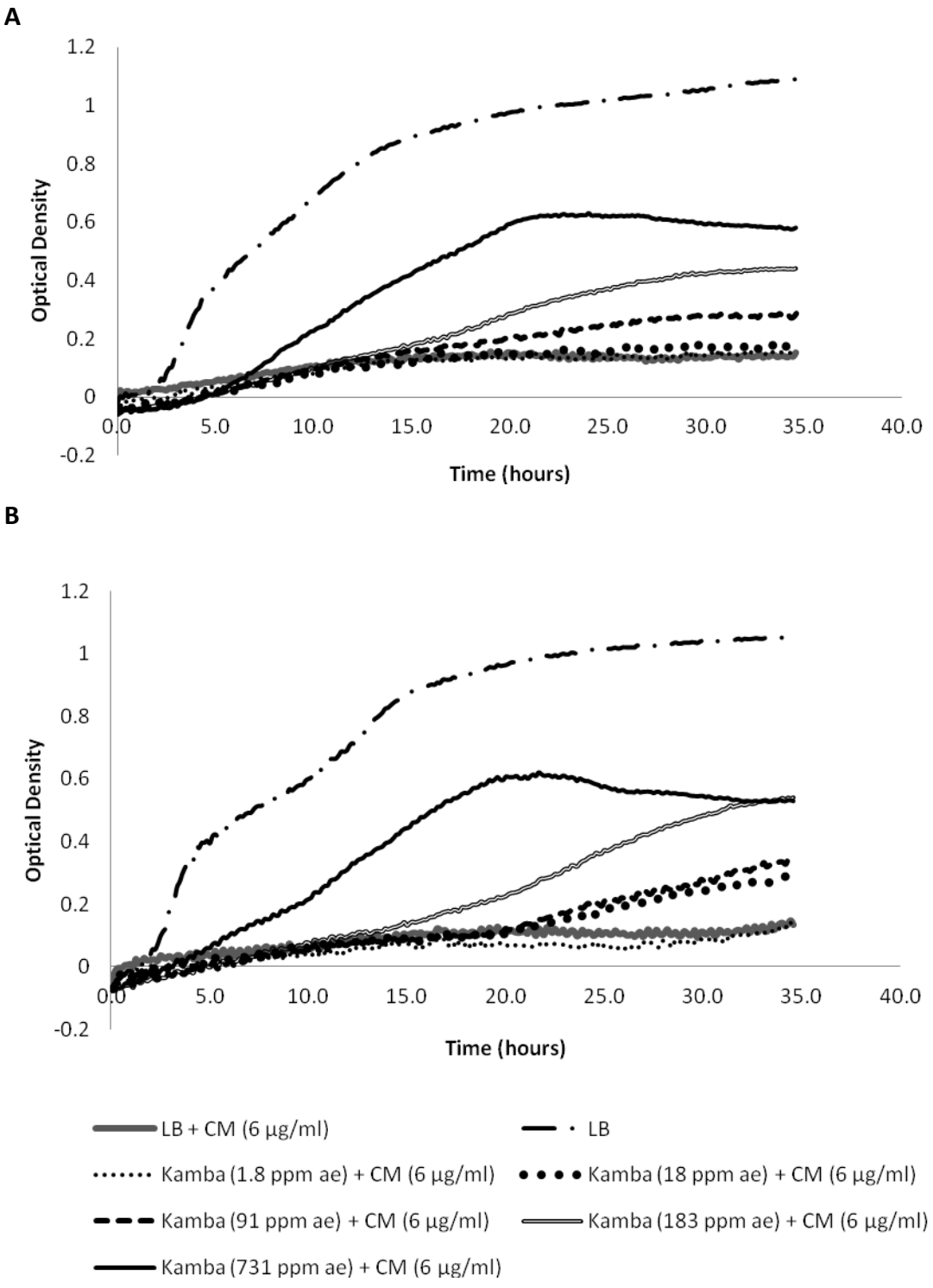

Figure 4.4. Effect on chloramphenicol tolerance in *S. typhimurium* pre-treated with two concentrations of Kamba.

Bacteria were pre-treated with 7310 ppm ae Kamba **(A)** and 10965 ppm ae Kamba **(B)**. Cells were then incubated in LB, chloramphenicol (CM) (6 µg/ml) and CM (6 µg/ml) plus Kamba (1.8, 18, 91, 183 and 731 ppm ae). Results are expressed as optical density measured over time, mean of three independent experiments.

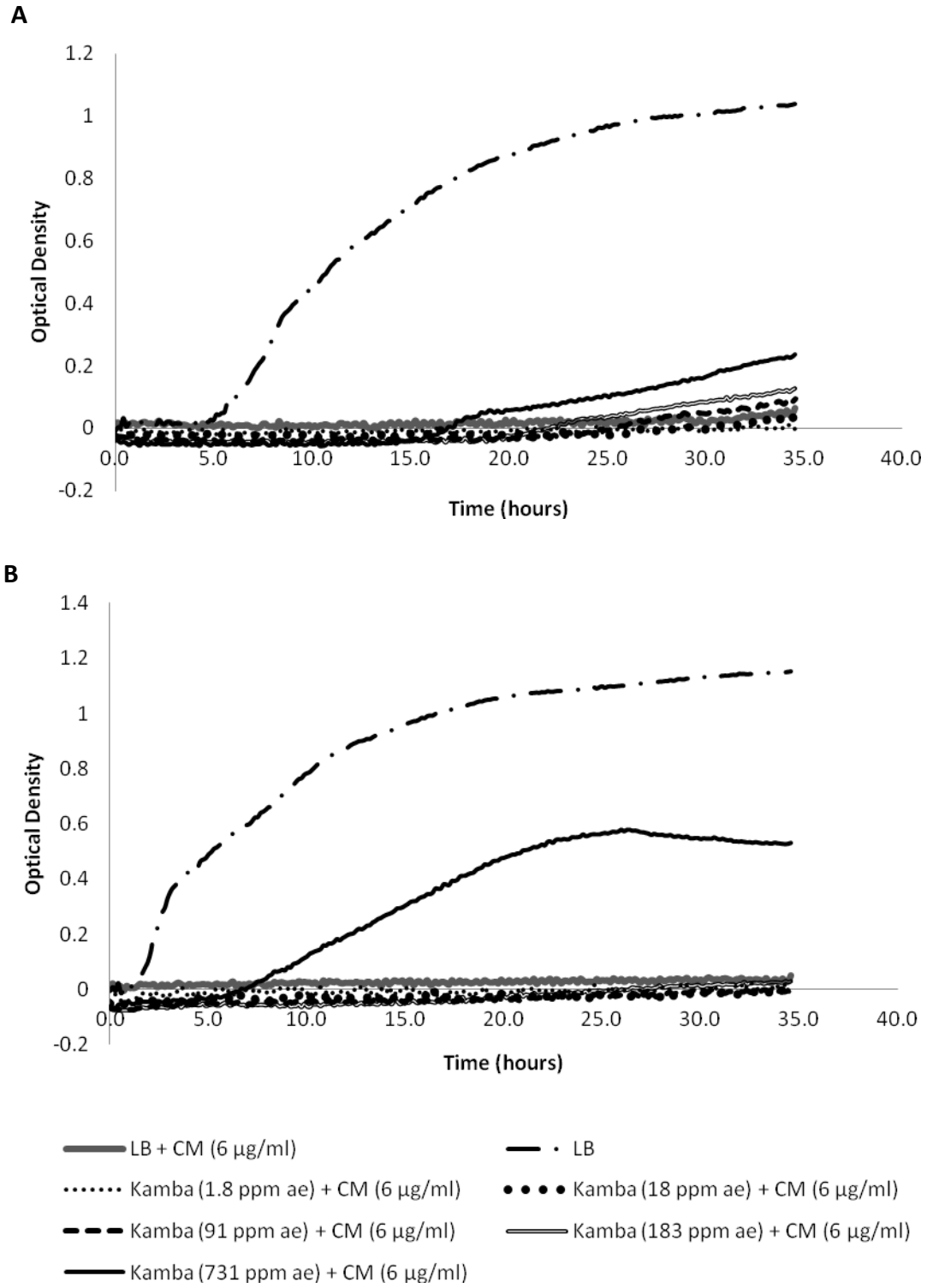

Figure 4.5. Effect on chloramphenicol tolerance in *S. typhimurium* pre-treated with chloramphenicol and no chemical treatment.

Bacteria were pre-treated with chloramphenicol (6 µg/ml) **(A)** and no chemical treatment **(B)**. Cells were then incubated in LB, chloramphenicol (CM) (6 µg/ml) and CM (6 µg/ml) plus Kamba (1.8, 18, 91, 183 and 731 ppm ae). Results are expressed as optical density measured over time, mean of three independent experiments.

4.3.2.2 Tetracycline

S. typhimurium pre-treated with tetracycline plus Kamba (731 ppm ae) was able to maintain tolerance at Kamba concentrations below induction level and also in its absence (Fig. 4.6 A). Unlike the maintenance of chloramphenicol tolerance in the absence of Kamba, there was a lag time before tetracycline tolerance was seen and the optical density was much lower.

Tetracycline tolerance was not maintained at below induction concentrations of Kamba and in its absence when bacteria were pre-treated with 731 ppm ae (Fig. 4.6 B), 7310 ppm ae (Fig. 4.7 A) and 10965 ppm ae (Fig. 4.7 B) Kamba. This is similar to the results seen with chloramphenicol. As expected, *S. typhimurium* was able to grow in media with Kamba (731 ppm ae) above induction concentration. The optical density for this condition was higher when bacteria were pre-treated with 10965 ppm ae and 7310 ppm ae compared to pre-treatment with 731 ppm ae, reconfirming the positive correlation between Kamba concentration and antibiotic response (Chapter 3, section 3.3.1). Tolerance was not maintained when *S. typhimurium* was pre-treated with tetracycline-only (Fig. 4.8 A) and when cells were not pre-treated (Fig. 4.8 B). This suggests that induction of tetracycline tolerance requires both Kamba and tetracycline.

When the final optical densities of each incubation condition (e.g. tetracycline plus 1.8 ppm ae Kamba) were compared between pre-treatments, none of the conditions were significantly different; the p-values were above the 0.01 threshold (Table 4.2). However, compared to the p-values at the start of incubation (Time = 0) the p-values after incubation decreased for most conditions except the tetracycline-only and no-chemical treatment. The lack of reduction in p-value for the tetracycline-only condition may be because pre-treatment with tetracycline and 731 ppm ae Kamba kept the optical densities low compared to the no-chemical treatment.

Table 4.2. p-values for the maintenance of tetracycline tolerance induced by Kamba

Condition	p-value	
	Time = 0 hours	**Time = 34 hours 35 minutes**
Tetracycline-only	0.016	0.044
No chemical treatment	0.07	0.749
Tetracycline + Kamba (1.8 ppm ae)	0.339	0.059
Tetracycline + Kamba (18 ppm ae)	0.845	0.03
Tetracycline + Kamba (183 ppm ae)	0.932	0.025
Tetracycline + Kamba (366 ppm ae)	0.784	0.036
Tetracycline + Kamba (731 ppm ae)	0.858	0.014

p-values before (time = 0) or after incubation (time = 34 hours 35 minutes), by the Kruskal-Wallis test.

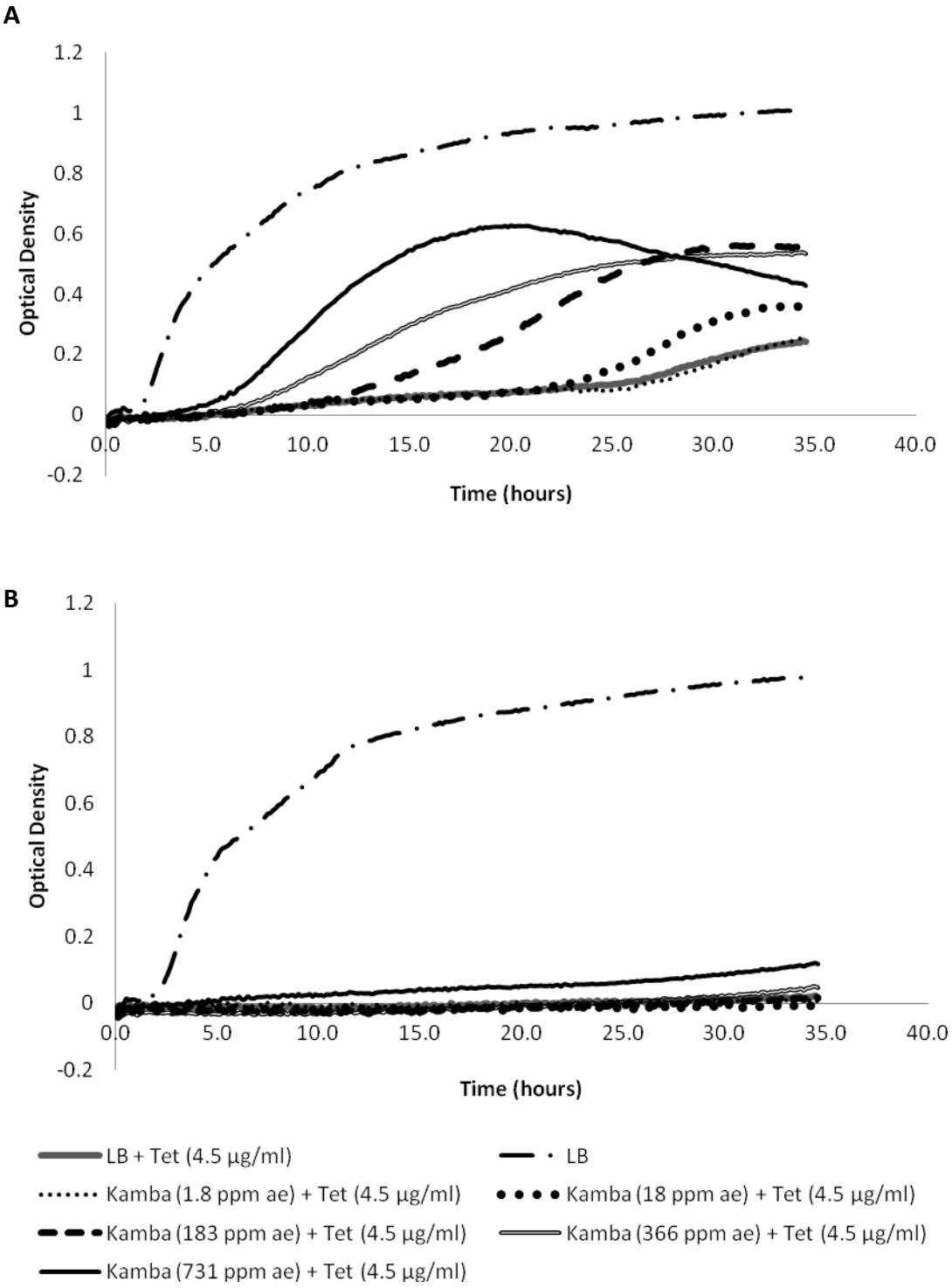

Figure 4.6. Effect on tetracycline tolerance in *S. typhimurium* pre-treated with tetracycline plus Kamba and Kamba-only.

Bacteria were pre-treated with tetracycline (4.5 µg/ml) plus Kamba (731 ppm ae) **(A)** and Kamba-only (731 ppm ae) **(B)**. Cells were then incubated in LB, tetracycline (Tet) (4.5 µg/ml) and Tet (4.5 µg/ml) plus Kamba (1.8, 18, 183, 366 and 731 ppm ae). Results are expressed as optical density measured over time, mean of three independent experiments.

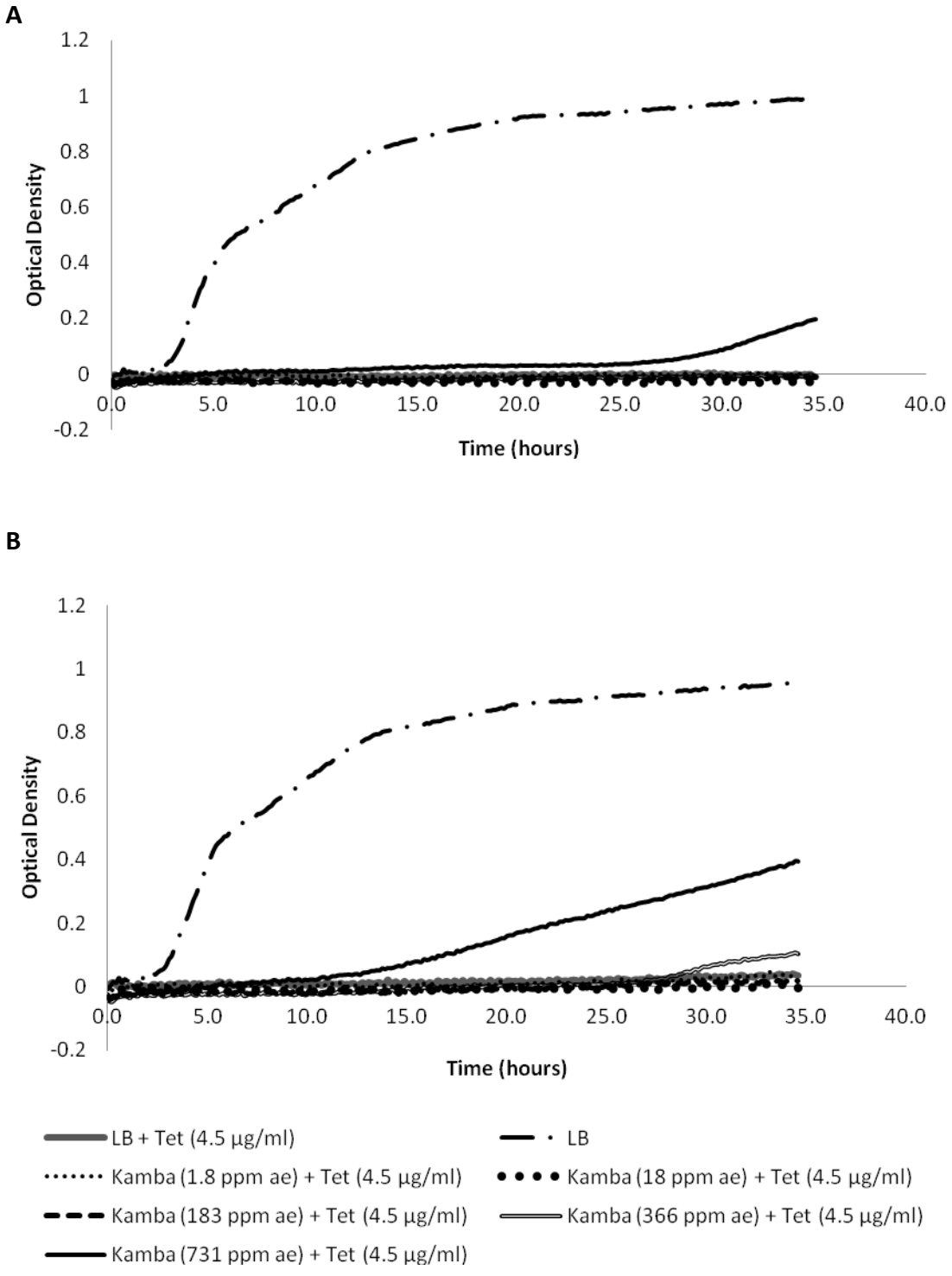

Figure 4.7. Effect on tetracycline tolerance in *S. typhimurium* pre-treated with two concentrations of Kamba.

Bacteria were pre-treated with 7310 ppm ae Kamba **(A)** and 10965 ppm ae Kamba **(B)**. Cells were then incubated in LB, tetracycline (Tet) (4.5 µg/ml) and Tet (4.5 µg/ml) plus Kamba (1.8, 18, 183, 366 and 731 ppm ae). Results are expressed as optical density measured over time, mean of three independent experiments.

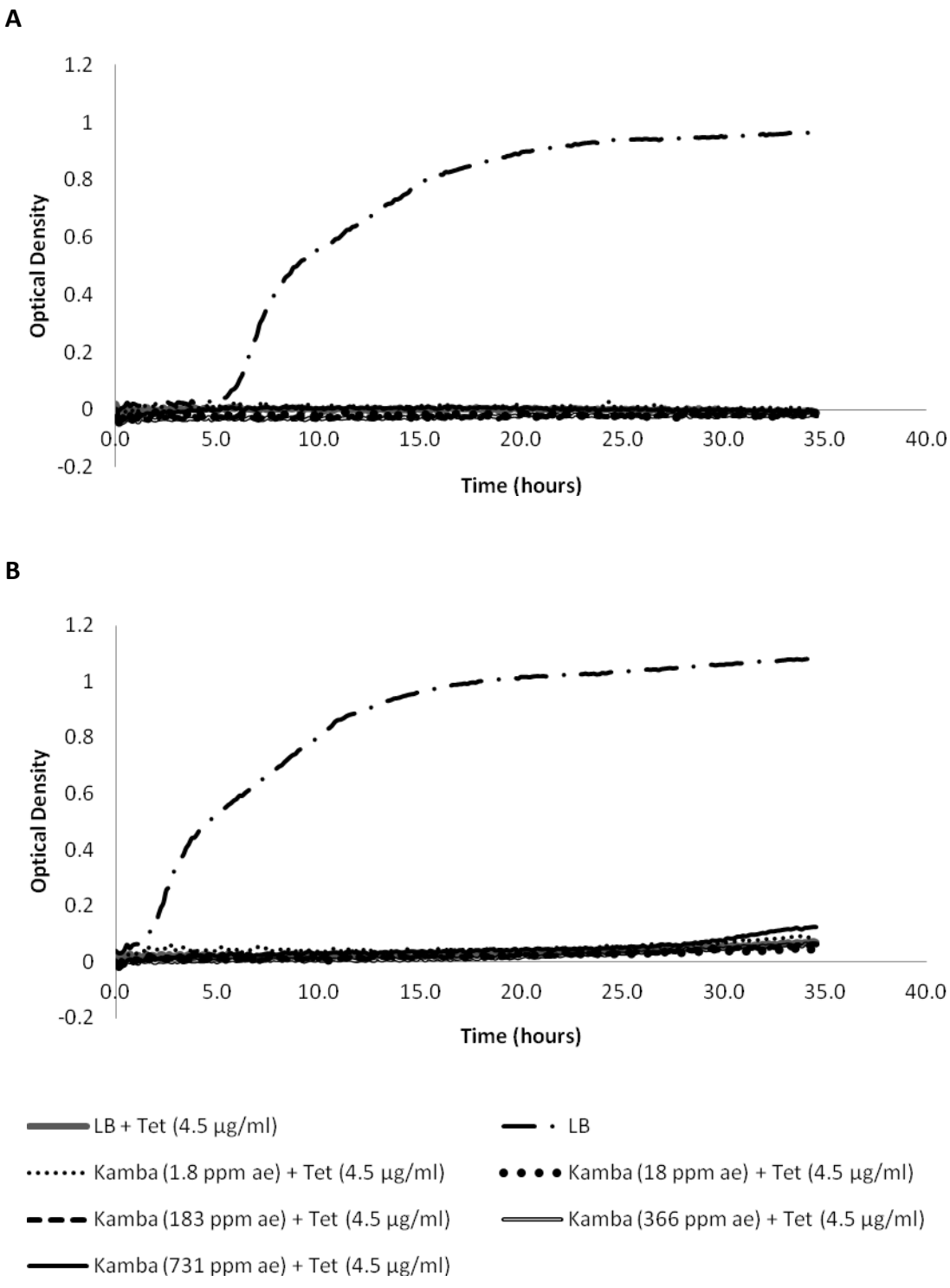

Figure 4.8. Effect on tetracycline tolerance in *S. typhimurium* pre-treated with tetracycline and no chemical treatment.

Bacteria were pre-treated with tetracycline (4.5 µg/ml) **(A)** and no chemical treatment **(B)**. Cells were then incubated in LB, tetracycline (Tet) (4.5 µg/ml) and Tet (4.5 µg/ml) plus Kamba (1.8, 18, 183, 366 and 731 ppm ae). Results are expressed as optical density measured over time, mean of three independent experiments.

4.3.2.3 Ciprofloxacin

Ciprofloxacin tolerance was maintained at below induction concentrations and in the absence of Kamba when *S. typhimurium* was pre-treated with ciprofloxacin plus Kamba (731 ppm ae) (Fig. 4.9 A). However, pre-treatment with only ciprofloxacin also caused tolerance even in the absence of Kamba (Fig. 4.11 A). This may be due to selection of incrementally more ciprofloxacin resistant mutants during pre-treatment. The tolerance seen in cultures pre-treated with chloramphenicol and Kamba (731 ppm ae) may be due to the selection of chloramphenicol tolerant cells by chloramphenicol alone. However, in the previous assay (Chapter 2, section 2.3.3) when bacteria were plated directly onto media with ciprofloxacin (0.03–0.05 µg/ml) there was no growth. In contrast, when Kamba was included in the plate media bacteria remained viable.

Parallel to the results of chloramphenicol and tetracycline tolerance, *S. typhimurium* exposed to 731 ppm ae (Fig. 4.9 B), 7310 ppm ae (Fig. 4.10 A) and 10965 ppm ae (Fig. 4.10 B) Kamba did not maintain ciprofloxacin tolerance in the absence of Kamba. *S. typhimurium* with no pre-treatment also did not maintain ciprofloxacin tolerance (Fig. 4.11 B).

When the optical densities at the endpoint of incubation for each incubation condition were compared between pre-treatments, the conditions were only marginally significant at the 0.01 p-value threshold (Table 4.3), suggesting that there may be a difference between pre-treatments after incubation for each treatment (except no-chemical treatment). Compared to the p-values at the start of incubation (Time = 0) the p-values after incubation decreased for the ciprofloxacin plus Kamba conditions.

Table 4.3. p-values for the maintenance of ciprofloxacin tolerance induced by Kamba

Condition	p-value	
	Time = 0 hours	Time = 34 hours 35 minutes
Ciprofloxacin-only	0.01	0.016
No chemical treatment	0.045	0.612
Ciprofloxacin + Kamba (1.8 ppm ae)	0.089	0.013
Ciprofloxacin + Kamba (18 ppm ae)	0.078	0.025
Ciprofloxacin + Kamba (183 ppm ae)	0.225	0.015
Ciprofloxacin + Kamba (366 ppm ae)	0.578	0.026
Ciprofloxacin + Kamba (731 ppm ae)	0.029	0.025

p-values before (time = 0) or after incubation (time = 34 hours 35 minutes), by the Kruskal-Wallis test.

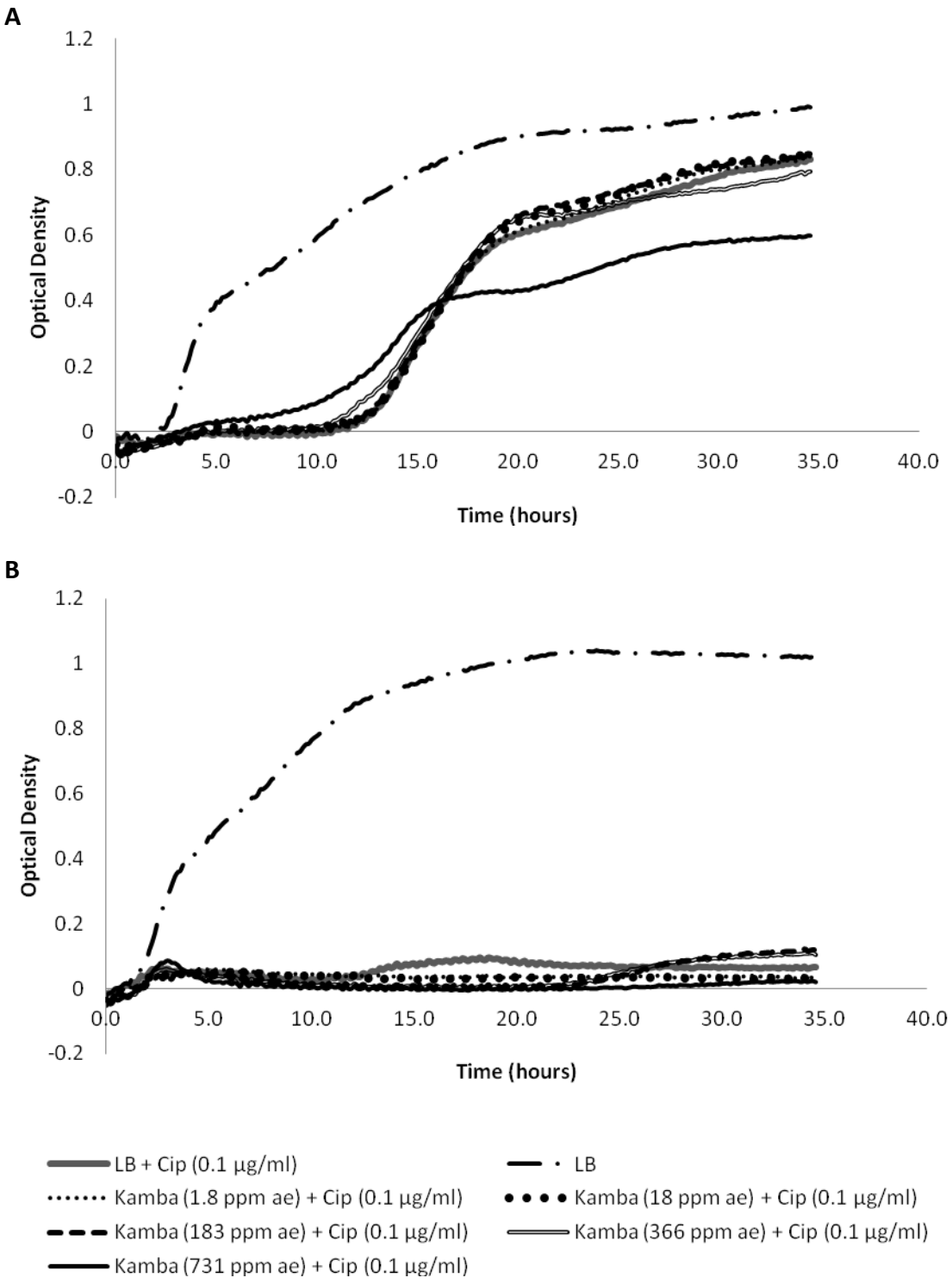

Figure 4.9. Effect on ciprofloxacin tolerance in *S. typhimurium* pre-treated with ciprofloxacin plus Kamba and Kamba-only.

Bacteria were pre-treated with ciprofloxacin (0.1 µg/ml) plus Kamba (731 ppm ae) **(A)** and Kamba-only (731 ppm ae) **(B)**. Cells were then incubated in LB, ciprofloxacin (Cip) (0.1 µg/ml) and Cip (0.1 µg/ml) plus Kamba (1.8, 18, 183, 366 and 731 ppm ae). Results are expressed as optical density measured over time, mean of three independent experiments.

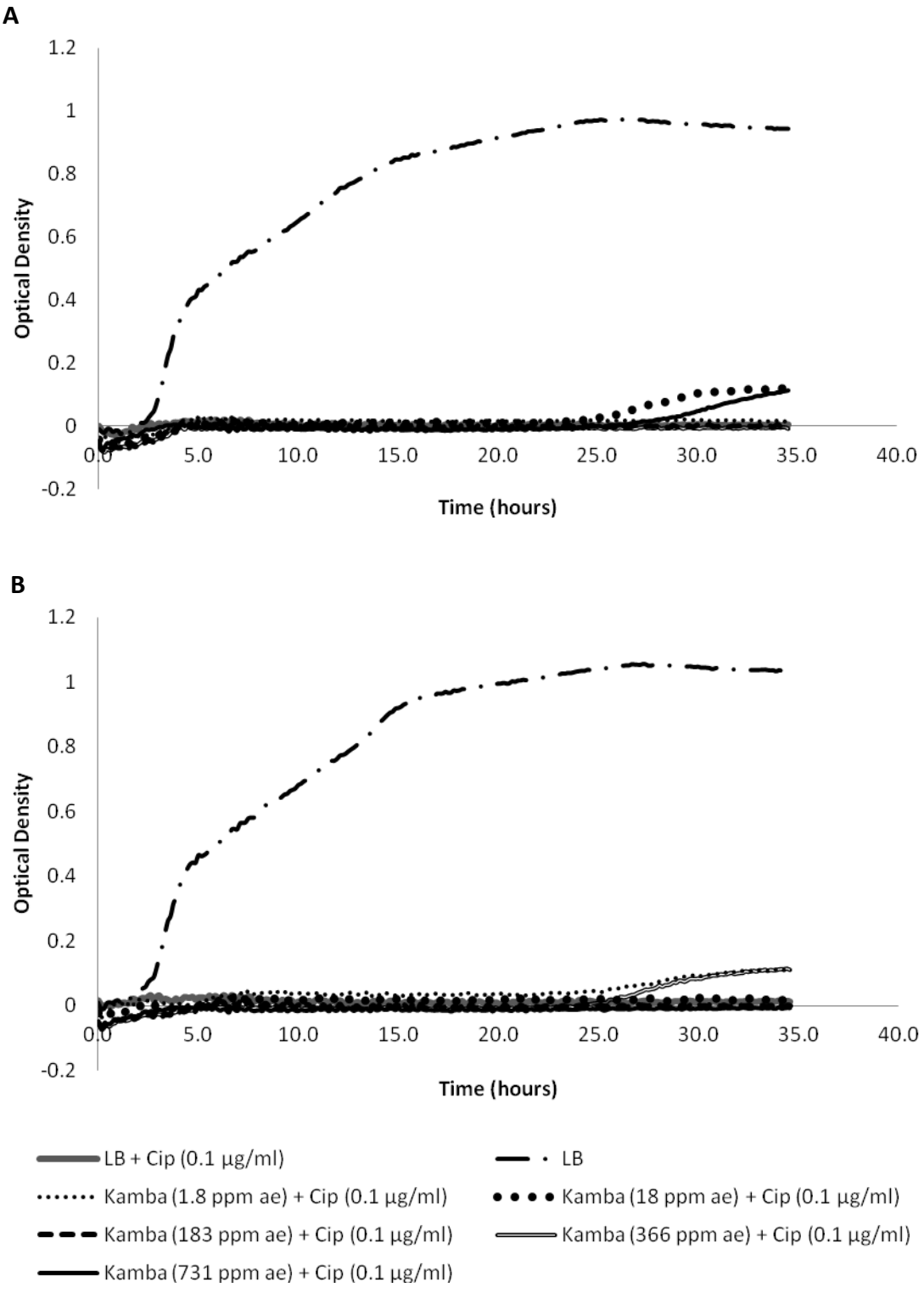

Figure 4.10. Effect on ciprofloxacin tolerance in *S. typhimurium* pre-treated with two Kamba concentrations.

Bacteria were pre-treated with 7310 ppm ae Kamba **(A)** and 10965 ppm ae Kamba **(B)**. Cells were then incubated in LB, ciprofloxacin (Cip) (0.1 µg/ml) and Cip (0.1 µg/ml) plus Kamba (1.8, 18, 183, 366 and 731 ppm ae). Results are expressed as optical density measured over time, mean of three independent experiments.

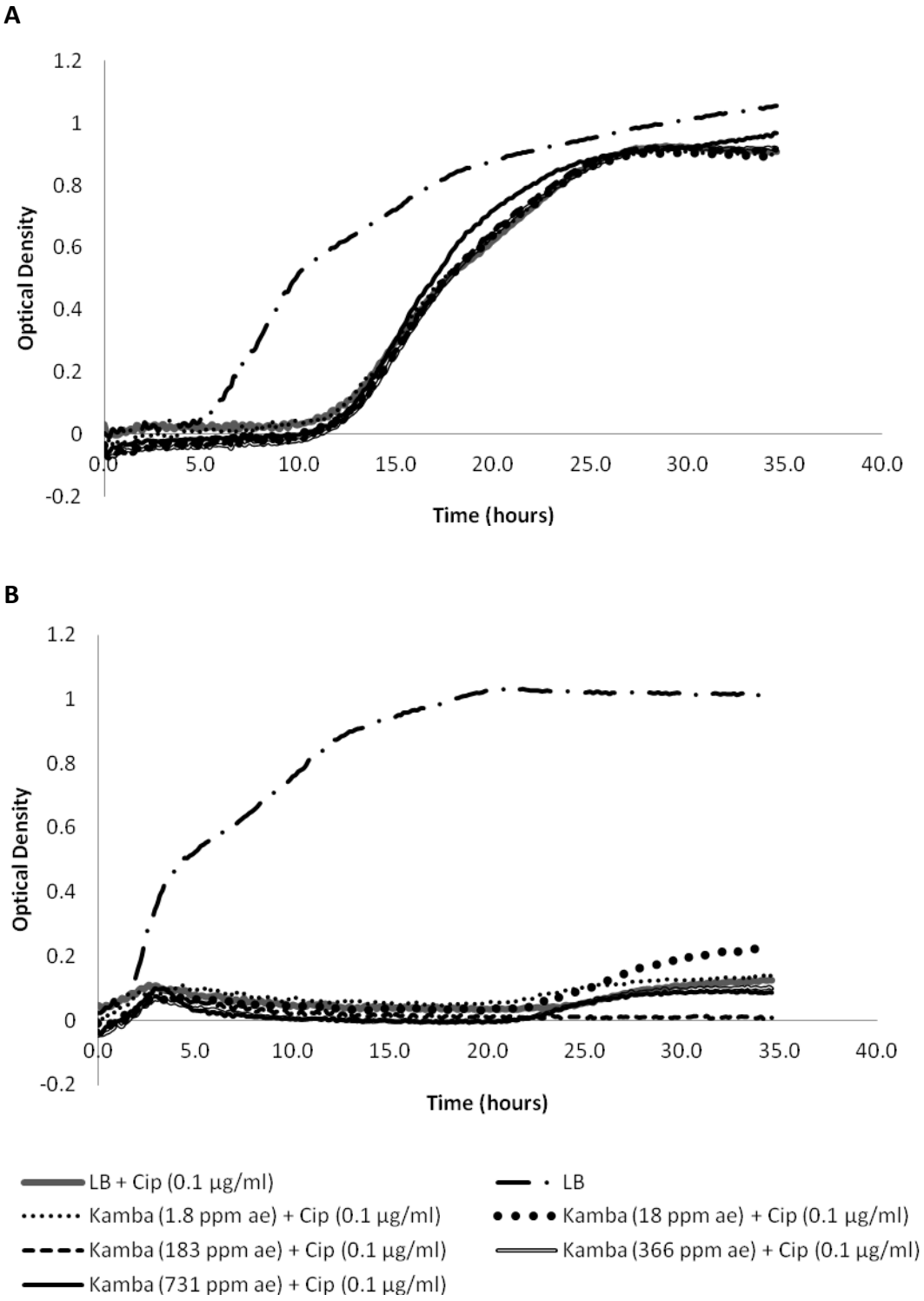

Figure 4.11. Effect on ciprofloxacin tolerance in *S. typhimurium* pre-treated with ciprofloxacin and no chemical treatment.

Bacteria were pre-treated with ciprofloxacin (0.1 µg/ml) **(A)** and no chemical treatment **(B)**. Cells were then incubated in LB, ciprofloxacin (Cip) (0.1 µg/ml) and Cip (0.1 µg/ml) plus Kamba (1.8, 18, 183, 366 and 731 ppm ae). Results are expressed as optical density measured over time, mean of three independent experiments.

4.3.2.4 Ampicillin

S. typhimurium pre-treated with ampicillin plus Kamba (731 ppm ae) was tolerant to ampicillin at Kamba concentrations below those needed for induction and this tolerance was also maintained in the absence of Kamba (Fig. 4.12 A). *S. typhimurium* pre-treated with 731 ppm ae (Fig. 4.12 B), 7310 ppm ae (Fig. 4.13 A) and 10965 ppm ae (Fig. 4.13 B) Kamba, were not tolerant to ampicillin in the absence or in the presence of Kamba at below induction concentrations. This suggests that exposure to Kamba alone is not sufficient to induce ampicillin tolerance.

Unlike the effect of ciprofloxacin on tolerance, pre-exposure to ampicillin-only did not elicit ampicillin tolerance (Fig. 4.14 A). *S. typhimurium* without pre-treatment also did not maintain tolerance (Fig. 4.14 B).

The final optical densities of each incubation condition (e.g. ampicillin plus 1.8 ppm ae Kamba) were compared between pre-treatments using the Kruskal-Wallis test. The ampicillin conditions with or without Kamba were only marginally significant when the pre-treatments were compared with each other at the p-value less than 0.01 threshold (Table 4.4). On the other hand, the no chemical treatment was not significant (p-value = 0.454). When the p-values after incubation were compared to the p-values at the start of incubation (Time = 0), the p-values decreased for all conditions except the no-chemical treatment, suggesting that there may be a greater difference between pre-treatments after incubation.

Table 4.4. p-values for the maintenance of ampicillin tolerance induced by Kamba

Condition	p-value	
	Time = 0 hours	Time = 34 hours 35 minutes
Ampicillin-only	0.128	0.037
No chemical treatment	0.166	0.454
Ampicillin + Kamba (1.8 ppm ae)	0.695	0.027
Ampicillin + Kamba (18 ppm ae)	0.791	0.016
Ampicillin + Kamba (183 ppm ae)	0.457	0.045
Ampicillin + Kamba (366 ppm ae)	0.788	0.031
Ampicillin + Kamba (731 ppm ae)	0.793	0.048

p-values before (time = 0) or after incubation (time = 34 hours 35 minutes), by the Kruskal-Wallis test.

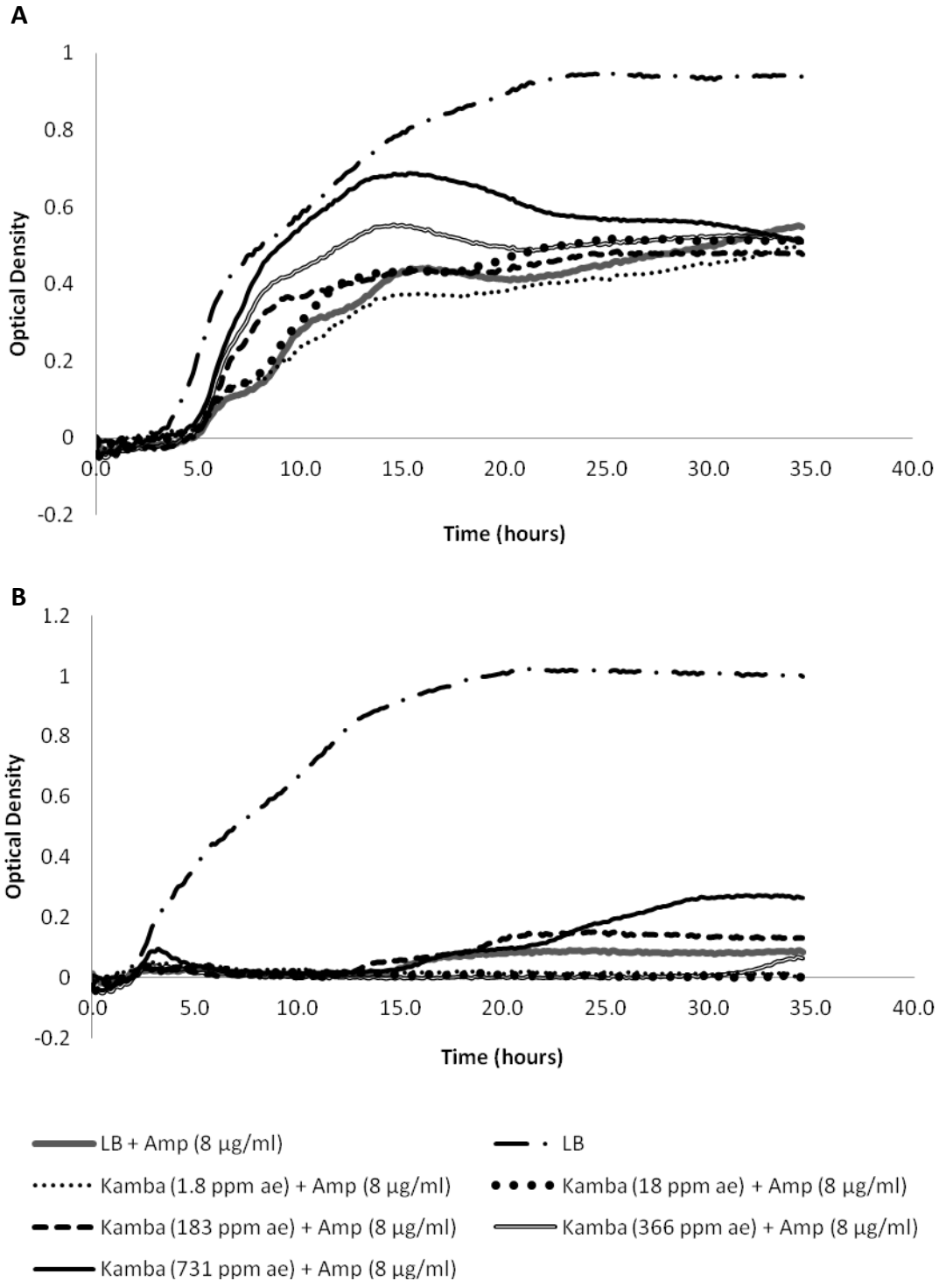

Figure 4.12. Effect on ampicillin tolerance in *S. typhimurium* pre-treated with ampicillin plus Kamba and Kamba-only.

Bacteria were pre-treated with ampicillin (8 µg/ml) plus Kamba (731 ppm ae) **(A)** and Kamba-only (731 ppm ae) **(B)**. Cells were then incubated in LB, ampicillin (Amp) (8 µg/ml) and Amp (8 µg/ml) plus Kamba (1.8, 18, 183, 366 and 731 ppm ae). Results are expressed as optical density measured over time, mean of three independent experiments.

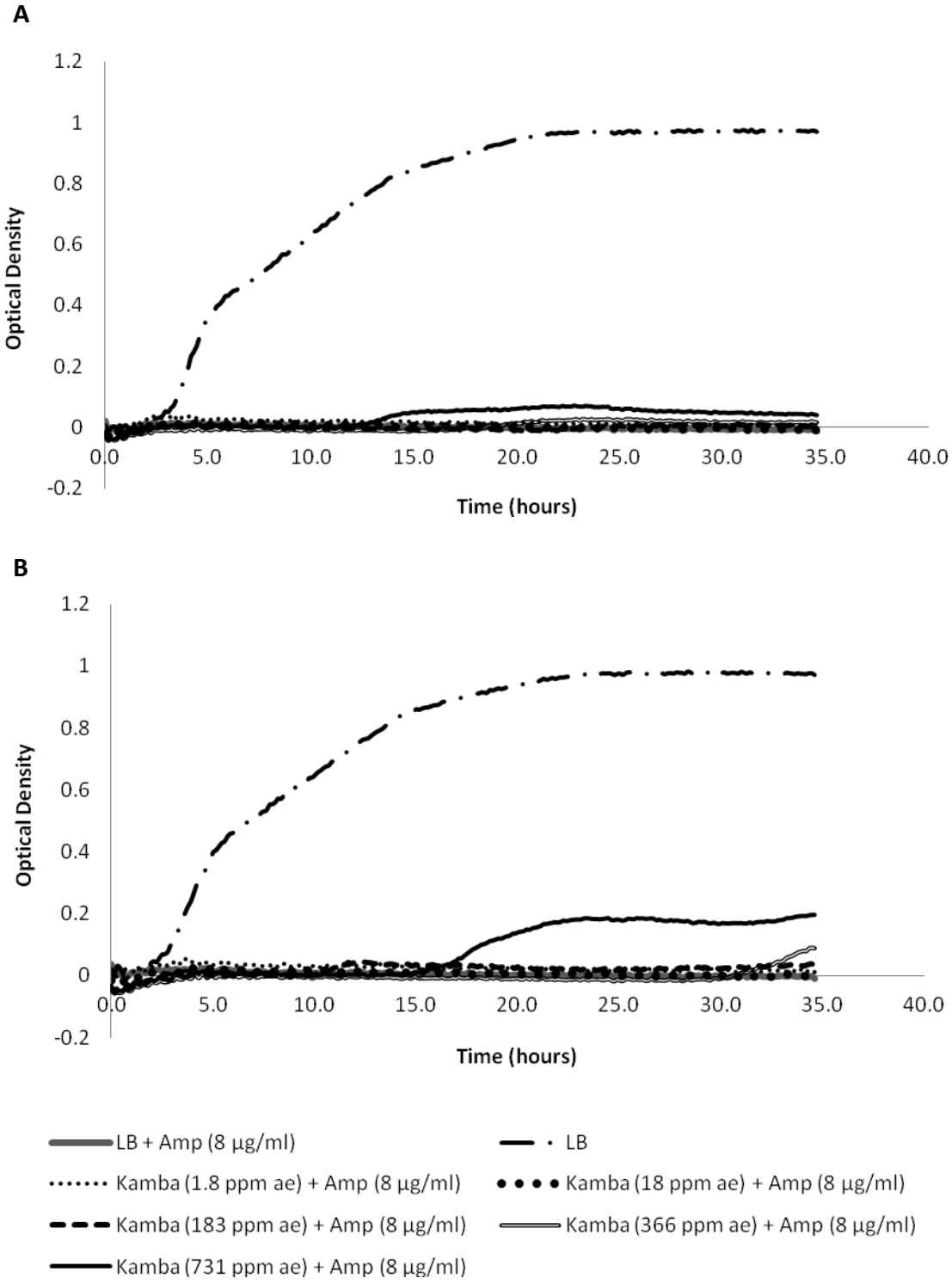

Figure 4.13. Effect on ampicillin tolerance in *S. typhimurium* pre-treated with two concentrations of Kamba.

Bacteria were pre-treated with 7310 ppm ae Kamba **(A)** and 10965 ppm ae Kamba **(B)**. Cells were then incubated in LB, ampicillin (Amp) (8 µg/ml) and Amp (8 µg/ml) plus Kamba (1.8, 18, 183, 366 and 731 ppm ae). Results are expressed as optical density measured over time, mean of three independent experiments.

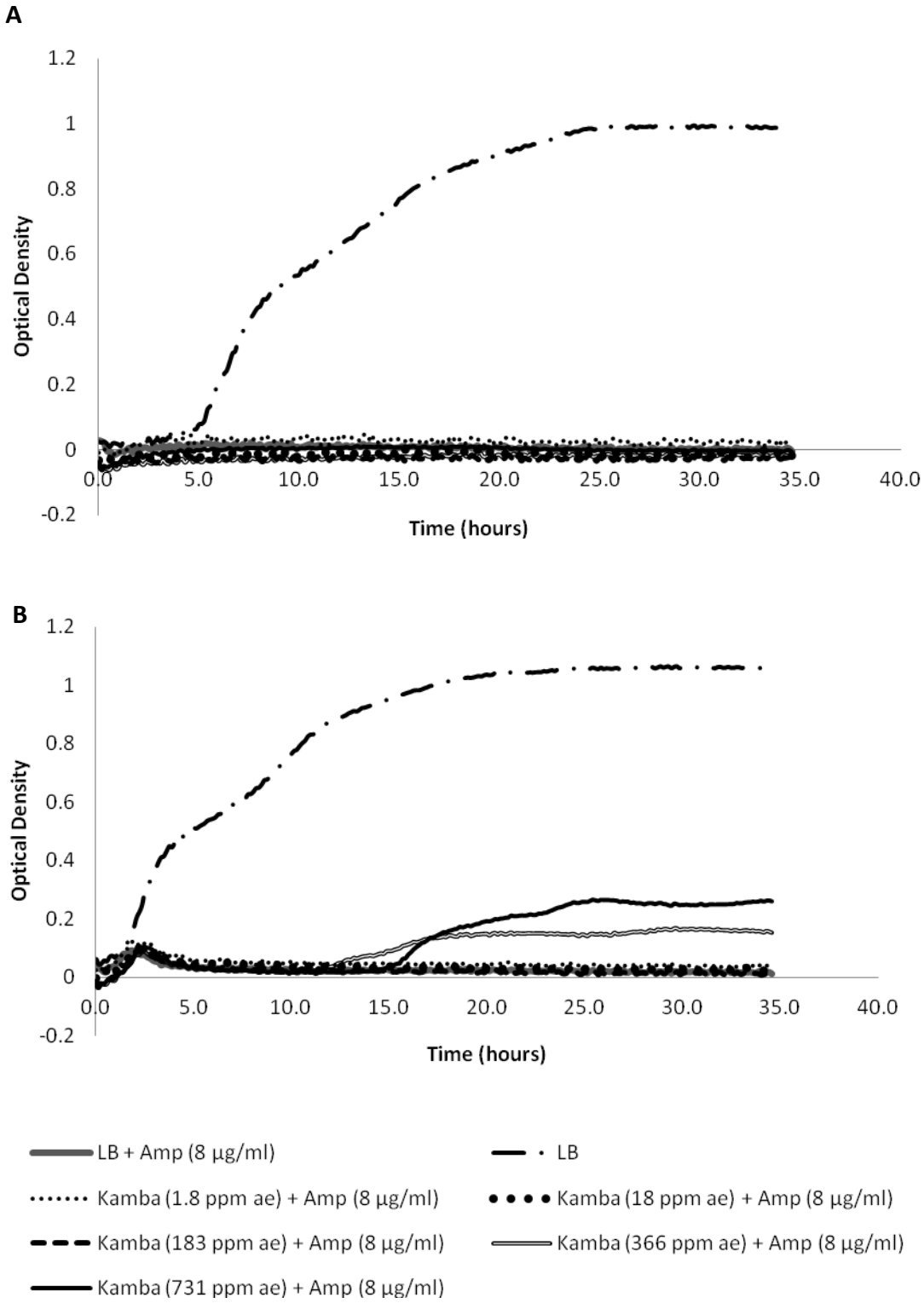

Figure 4.14. Effect on ampicillin tolerance in *S. typhimurium* pre-treated with ampicillin-only and no chemical treatment.

Bacteria were pre-treated with ampicillin (8 µg/ml) **(A)** and no chemical treatment **(B)**. Cells were then incubated in LB, ampicillin (Amp) (8 µg/ml) and Amp (8 µg/ml) plus Kamba (1.8, 18, 183, 366 and 731 ppm ae). Results are expressed as optical density measured over time, mean of three independent experiments.

4.3.2 The effect of herbicide mixtures on antibiotic response

The combined effects of herbicides on antibiotic response toward chloramphenicol and kanamycin were studied. *S. typhimurium* was grown in the herbicide combinations: Kamba plus Roundup and Kamba plus 2,4-D. Following incubation, the cultures were examined visually for cloudiness which indicated bacterial growth. Chloramphenicol was chosen to represent antibiotics for which *S. typhimurium* had increased tolerance in the presence of Kamba and 2,4-D. Kanamycin was chosen to represent antibiotics for which *S. typhimurium* had decreased tolerance upon exposure to Kamba and 2,4-D.

In the herbicide combinations, both herbicides contributed to a change in the MIC of chloramphenicol and kanamycin. For chloramphenicol MIC, the presence of Kamba and 2,4-D increased the MIC from 5 μg/ml chloramphenicol to > 8 μg/ml and 7 μg/ml, respectively. The presence of Roundup decreased the MIC to < 1.6 μg/ml (Table 4.5). For *S. typhimurium* exposed simultaneously to both Kamba and Roundup the MIC decreased to 3.3 μg/ml, just below the wild type (antibiotic-only) MIC (5μg/ml) but above the MIC of the Roundup-only condition (< 1.6 μg/ml). For *S. typhimurium* exposed to both Kamba and 2,4-D the MIC increased to 7.3 μg/ml (Table 4.5). However, the increase in MIC was not greater than the increase seen in the Kamba-only condition (> 8 μg/ml).

Simultaneous exposure of *S. typhimurium* to Kamba (an herbicide that decreases kanamycin tolerance) and Roundup (an herbicide that increases kanamycin tolerance) caused a decrease in tolerance (Table 4.5). The MIC decreased to 5.3 μg/ml, just above the MIC of the Kamba treatment (4 μg/ml). The results suggest that the Kamba effect was more powerful than the Roundup effect.

Kanamycin tolerance was also decreased when *S. typhimurium* was exposed to Kamba and 2,4-D (Table 4.5). The MIC decreased to 8 μg/ml, which was between the MIC of Kamba and 2,4-D conditions individually.

Table 4.5. Minimum antibiotic concentrations that inhibit *S. typhimurium* under different herbicide treatments

Herbicide	Antibiotic	
	Chloramphenicol (µg/ml)	Kanamycin (µg/ml)
No herbicide	5.0 ±1.0	20 ±0.0
Kamba	>8.0 ±0.0	4.0 ±0.0
Roundup	<1.6 ±0.0	>20 ±0.0
2,4-D	7.0 ±0.0	12 ±0.0
Kamba + Roundup	3.3 ±0.3	5.3 ±1.3
Kamba + 2,4-D	7.3 ±0.3	8.0 ±0.0

MICs ± SEM (n=3) were measured in liquid broth supplemented with herbicide combinations in 24 well plates. Bacterial growth was determined visually following incubation at 37^0C for 16-20 hours. Kamba - 1827 ppm ae, Roundup - 1243 ppm ae and 2,4-D - 1940 ppm ae.

4.4 Discussion

Results from the current study have shown that the herbicides: Kamba, 2,4-D and Roundup induced an antibiotic response in *S. typhimurium* (Chapter 2). The herbicide concentrations necessary to induce an effect were dependent on the antibiotic and herbicide, and some were within the MRLs (Chapter 3). Nonetheless, the minimum induction concentrations for all herbicides were within the application rates recommended by manufacturers.

Here, the maintenance of antibiotic tolerance once induced by Kamba was determined. In addition, the effect on antibiotic response with Kamba-only treatment was studied. *S. typhimurium* was first pre-treated in three conditions: antibiotic, antibiotic plus Kamba and Kamba-only, and was then grown in media with antibiotic and antibiotic with increasing amounts of Kamba. The aim of this assay was to evaluate whether the induced antibiotic tolerance can be maintained at lower Kamba concentrations (within MRLs) or in the absence of Kamba.

The effect of dual herbicides on antibiotic response was also studied. The change in chloramphenicol and kanamycin tolerance in the presence of two herbicide combinations was determined. The antibiotic MICs were determined for *S. typhimurium* exposed to the herbicide combinations in liquid media and were compared against the antibiotic MICs when *S. typhimurium* was exposed to only one herbicide.

4.4.1 Maintenance of antibiotic tolerance

In this study, *S. typhimurium* pre-treated with only Kamba did not show maintenance of antibiotic tolerance either on plates or in liquid media. Three concentrations of Kamba were tested – 731, 7310 and 10965 ppm ae - and all were above the minimum inducing concentrations reported in Chapter 3. The highest amount of Kamba used in this assay (10965 ppm ae) was close to the MIC of Kamba (14485 ppm ae) and the bacteria in this condition were growing slower than normal, hence even higher concentrations of Kamba were not tested.

Because lower concentrations of Kamba were sufficient to induce a response in the presence of antibiotic, it was initially thought that the cells may require the antibiotic and herbicide to reach a threshold, above which tolerance is induced. Hence, a higher concentration of Kamba may be able to overcome this threshold. However, the results gathered here suggest that the antibiotic response is induced only in the presence of herbicide and antibiotic.

Maintenance of chloramphenicol tolerance was tested both on plates (solid media) and using the plate reader (liquid media). The assay on solid media allowed us to calculate the proportion of cells that were viable due to the herbicide. In addition, the chloramphenicol tolerant colonies could be re-tested for prolonged (5 days) tolerance. The assay in liquid media monitored the growth of bacterial cells over time and determined if there was a lag time. Furthermore, it helped determine if the growth of the culture would plateau after a certain time possibly due to limited nutrients. This method enabled us to run a larger number of samples, however, maintenance of tolerance over 5 days could not be tested. The results gathered in both assays showed similar trends in chloramphenicol tolerance. Therefore, only the liquid assay was used for testing maintenance of tolerance to tetracycline, ciprofloxacin and ampicillin.

S. typhimurium pre-treated with antibiotic (above MIC) and Kamba (above inducing concentration) maintained tolerance towards chloramphenicol, tetracycline and ampicillin. *S. typhimurium* not only remained tolerant when grown in Kamba below the minimum inducing concentration, but even in absence of the herbicide. Hence, the antibiotic response observed here first requires the presence of both the herbicide and antibiotic and can then be maintained in the absence of herbicide.

Ciprofloxacin tolerance was maintained when bacteria were pre-treated with ciprofloxacin, and ciprofloxacin plus Kamba. The antibiotic response seen may be due to the effect of only ciprofloxacin. It is well known that exposure to an antibiotic can select for sub-populations that are more tolerant to the antibiotic (Wiuff *et al.*, 2005). It is possible that pre-treatment with ciprofloxacin selected for tolerant cells which may have subsequently increased the optical density readings. In Chapter 2, tolerant colonies on the ciprofloxacin-only plates were not observed in the assay that initially showed herbicide-induced ciprofloxacin tolerance, while

colonies grew on the ciprofloxacin plus Kamba plates. Due to tolerance not being maintained in the Kamba-only and no-treatment conditions we can be sure that the ciprofloxacin concentration used in this assay was above MIC. Therefore, the tolerance maintained in cells pre-treated with ciprofloxacin may be due to a sub-population that was tolerant and selected for during pre-treatment.

The initial study done by Rosner (1985) and subsequent studies (Cohen *et al.*, 1993; Berlanga & Vinas, 2000) showed that salicylic acid-induced antibiotic tolerance occurred only when cultures were exposed to salicylic acid and antibiotic simultaneously. The results gathered here are consistent with these studies as *S. typhimurium* had to be exposed to herbicide and antibiotic in order to demonstrate a phenotypic antibiotic response.

Both herbicides and antibiotics are widely used in the environment. For over 50 years antibiotics have been used in medicine (Mackie *et al.*, 2006; Manzetti & Ghisi, 2014) and feed additives for animals (Esiobu *et al.*, 2002; Davis *et al.*, 2006). It is estimated that nearly 70% of the antibiotics manufactured annually are given to chickens, pigs and cows for non-therapeutic purposes and in agriculture to shield plants and fruits from diseases (Esiobu *et al.*, 2002).

Feeding antibiotics to animals is known to speedup growth and increase nutrient absorption and hence feed efficiency (Key & McBride, 2014). Tetracycline is one of the antibiotics commonly used in the honey bee (Valette *et al.*, 2004), pig and poultry industries (Mackie *et al.*, 2006). Antibiotics used as growth promoters in animals are poorly absorbed in the digestive tract and are eventually released into the environment (Zurek & Ghosh, 2014).

Antibiotics used in food production have been linked to increased antibiotic tolerant bacteria in animals and humans due to increased selection pressure (Esiobu *et al.*, 2002). A study comparing the number of antibiotic-tolerant bacteria found that there were significantly more tolerant bacteria in farm manure than other sites such as residential soil (Esiobu *et al.*, 2002).

Vectors that could create an environment for antibiotic-tolerant bacteria are insects. Multi-drug tolerant bacteria have been found in houseflies, cockroaches and honey bees (Zurek & Ghosh, 2014). Insects on farms may be simultaneously exposed to herbicides and antibiotics creating a

suitable environment for the development of antibiotic-tolerant bacteria. These tolerant bacteria may be transferred to food stuffs upon contact with insects (Zurek & Ghosh, 2014).

It is plausible that bacteria can come into contact with both herbicides and antibiotics simultaneously. Manure from farms with antibiotic fed livestock could be used on crop farms or pastures that use herbicides. Herbicide residues may be transferred to animal farms through the hay and grain used to feed livestock. The high application rates of herbicides may induce an antibiotic response in bacteria and as the results of this study show, the response may be maintained at lower herbicide concentrations or in the absence of herbicide.

On solid media, chloramphenicol tolerance that was induced during pre-treatment with chloramphenicol plus Kamba was maintained for 5 days. During this period the bacteria were serially plated on LB and were not exposed to Kamba. Only the bigger sized colonies from the cultures treated with chloramphenicol plus Kamba and subsequently plated on Kamba above inducing concentration, were able to maintain tolerance for 5 days. Exposure to Kamba during pre-treatment and plating may have given rise to two sub-populations, one that requires herbicide to maintain tolerance and one that does not. This may be due to selection of chloramphenicol resistant cells, however the colonies remained tolerant for 5 days and did not revert to susceptibility (over that time). Hence, the herbicide may have permitted the cells to achieve more generations of growth than cells exposed only to the antibiotic. Those additional generations may have been sufficient for one or more mutations relevant to chloramphenicol tolerance to arise. A study on salicylic acid and ciprofloxacin resistance in *Staphylococcus aureus*, found that salicylic acid not only allowed a greater number of cells to survive but also increased the mutation frequency of susceptible strains to higher ciprofloxacin tolerance levels (Gustafson *et al.*, 1999). Furthermore, following 3 passages on drug-free media, the *S. aureus* isolates grown on ciprofloxacin and salicylic acid had higher ciprofloxacin MICs compared to the parental strain (Gustafson *et al.*, 1999).

The data gathered here are contrary to the results found by Rosner (1985), where salicylic acid-induced chloramphenicol tolerance was not inherited but was only phenotypic (Rosner, 1985). The results are also contrary to those found by Novick *et al.,* (1957). In this famous

experiment the regulatory network of the *lac* operon was studied, and it was shown that the induced state of *E. coli* could be transferred to many generations at low inducer concentrations (Novick & Weiner, 1957; Vilar *et al.*, 2003). However, when pre-induced bacteria were transferred to medium without the inducer, the phenotype was lost (Novick & Weiner, 1957).

In the current study, *S. typhimurium* that was pre-treated in liquid media with herbicide and antibiotic, remained tolerant to chloramphenicol, tetracycline, ampicillin and ciprofloxacin in the absence of Kamba. The antibiotic that was present in the media may have acted as an inducer which maintained the observed effect. However, this does not explain the chloramphenicol tolerance maintained for 5 days (on plates), because bacteria were not exposed to either the herbicide or antibiotic. Preliminary experiments suggested that the chloramphenicol tolerant colonies were not tolerant to higher amounts of chloramphenicol, however, they may be able to gain incremental increases in tolerance through mutations.

The development of antibiotic-tolerant bacteria is of concern as it reduces the effectiveness of antibiotic therapy and may cause prolonged hospitalization and increased healthcare costs (Zurek & Ghosh, 2014). The maintenance of herbicide induced antibiotic response may interfere with antibiotic therapies in both humans and animals.

There is also a potential for these herbicides to cause increased tolerance in strains that are already tolerant to antibiotics. Rosner (1985) showed that a strain of *E. coli*, tolerant to higher amounts of chloramphenicol due to a mutation, had even higher tolerance to chloramphenicol when grown in the presence of salicylic acid (Rosner, 1985). Therefore, if bacteria that are already tolerant to antibiotics come into contact with herbicides they may develop tolerances to higher antibiotic concentrations.

Future experiments should include testing the maintenance of antibiotic response induced by 2,4-D and Roundup on plates which can give quantitative results. Maintenance of tolerance to higher antibiotic concentrations should also be tested. Here, only chloramphenicol tolerant colonies were re-streaked for 5 days. Colonies tolerant to other antibiotics should also be tested for prolonged tolerance. The chloramphenicol tolerant colonies that are of different sizes should be examined for differences in the activated efflux pumps, e.g., using quantitative

RT-PCR. It would be worth testing a strain of *S. typhimurium* that already has increased tolerance to an antibiotic, to see if exposure to herbicide will further increase its tolerance.

4.4.2 Herbicide combinations affect chloramphenicol and kanamycin tolerance

When herbicides were combined, each of the herbicides contributed to the antibiotic response. When *S. typhimurium* was exposed to two herbicides, one that increases tolerance and one that decreases tolerance, the overall antibiotic response was susceptibility. However, bacteria in this condition were not as susceptible when compared to cells that were incubated only with the herbicide that caused susceptibility.

When *S. typhimurium* were grown in media with two herbicides, both that increase tolerance, the overall antibiotic response was increased tolerance but the level of tolerance was not additive. Instead, the relative increase in tolerance was in between the response elicited by the herbicides individually. Similarly, exposure to two herbicides that decrease tolerance, the antibiotic response was susceptibility but the magnitude was between the responses seen in the individual herbicide treatments.

Due to the emergence of glyphosate-tolerant weeds, experts recommend using mixtures with different modes of action and metabolic pathways (Green *et al.*, 2008; Beckie, 2011). This eases the selection pressure on weeds to develop tolerance to a single herbicide. This is due to the action of the other herbicides in the spray mixture with other modes of action (Green *et al.*, 2008) which will prevent the growth of tolerant weeds (Green *et al.*, 2008). However, this may only temporarily halt the development of tolerant weeds (Baylis, 2000). Farmers have already reported weeds that are tolerant to more than five herbicides (Nature Editorials, 2014). Furthermore, there is concern that farmers will rely heavily on the herbicides for weed control, while abandoning integrated weed management strategies, which may result in weeds tolerant to multiple herbicides (Nature Editorials, 2014).

To manage the glyphosate-tolerant weed - *Conyza Canadensis,* farmers are supplementing glyphosate with other herbicides like 2,4-D and dicamba before planting glyphosate-tolerant

soybeans, corn and cotton (Beckie, 2011). It is vital that the herbicide concentrations used in the mixtures are at an optimum concentration in order to reduce the risk of multiple herbicide tolerant weeds.

A recent study on herbicide tolerance evolution found that doses of herbicide mixtures at or close to MIC were effective in delaying or preventing the evolution of herbicide tolerance (Lagator *et al.*, 2013). This study used a unicellular green chlorophyte as a model organism and one of the herbicides tested was glyphosate. Over time, the number of populations that evolved tolerance increased both in single and multiple herbicide treatments. However, tolerance evolved at a significantly slower rate when multiple herbicides were used (Lagator *et al.*, 2013). This illustrates that herbicide mixtures can only be a temporary fix (Nature Editorials, 2014).

Despite the increased need for novel approaches to effectively manage weeds, agrochemical companies have not had a great degree of success in discovering new herbicides with different modes of actions (Green *et al.*, 2008). In fact, companies have not brought out herbicides with new modes of action in the past 30 years (Heap, 2014a). Instead, most companies are marketing mixtures of herbicides that have been premixed with two or more active ingredients into a single formulation (Green *et al.*, 2008). New formulations include Enlist Duo®™ which contains glyphosate and 2,4-D as the active ingredients and Xtend which contains glyphosate and dicamba. Agrochemical companies are also pushing for the commercialization of crops with multiple tolerances such as soybeans with dicamba and glyphosate tolerance (Green *et al.*, 2008) which will further increase the use of herbicide mixtures.

The use of herbicide mixtures also increases the environmental herbicide load (Lagator *et al.*, 2013). Consequently, the amounts of herbicide residues that leach into soil and aquatic environments may increase. Bacteria exposed to herbicides are therefore likely to come into contact with two or more herbicides. The combined effects of herbicides on antibiotic response will depend on the particular herbicides.

Exposure to 2,4-D and Kamba, both herbicides that increase tolerance did not induce high level tolerance in *S. typhimurium*. Instead the induced tolerance was between the tolerances

induced by the herbicides individually. This suggests that the herbicides induce a response through the same mechanisms and pathways. Therefore, the magnitude of the antibiotic response may be limited by the availability of the active sites for herbicides.

Chapter Five

5. Summary and Future work

In this study, the effects of three commercial herbicide formulations (Kamba, 2,4-D and Roundup) on bacterial toxicity and tolerance to five antibiotics (chloramphenicol, tetracycline, ciprofloxacin, ampicillin and kanamycin) were investigated. The minimum concentration of herbicide that induced an antibiotic response was determined. Also, maintenance of the induced tolerance and the effects of herbicide combinations were studied. This study also attempted to answer whether the antibiotic response can be induced by herbicides present in water.

Antibiotic tolerance is an important health issue worldwide that compromises treatment of serious bacterial infections. Over the last two decades, there has been a rapid increase in multi-drug resistant pathogens, some that are resistant to most or virtually all antibiotics (Shea, 2003). The lack of new drugs is causing patients to face the prospect of untreatable infections (Shea, 2003; Key & McBride, 2014). If herbicides have an impact on antibiotic tolerance it would add to the current health care crisis as it may lead to even fewer effective antibiotics.

Salicylic acid has been shown to exert an antibiotic response in bacteria. *Escherichia coli* exposed to salicylic acid has increased tolerance towards tetracycline, chloramphenicol and ampicillin (Rosner, 1985; Cohen *et al.*, 1993). Salicylic acid also enhanced ciprofloxacin and nalidixic acid tolerance in *Serratia mercescens* (Berlanga & Vinas, 2000). Our initial notion was that the effect on antibiotic response may extend to other chemicals similar in structure to salicylic acid. Both dicamba and 2,4-D are structurally related to salicylic acid, which led us to question if these herbicides affect antibiotic tolerance in bacteria. *Salmonella enterica* serovar *Typhimurium* and *E. coli* were chosen as model organisms to investigate these hypotheses. The results for *S. typhimurium* were detailed in this report.

We determined the concentrations of herbicides that were toxic to *S. typhimurium*. More importantly we present the first evidence of an herbicide-induced antibiotic response in

S. typhimurium. Kamba and 2,4-D *increased* tolerance towards chloramphenicol, tetracycline, ciprofloxacin and ampicillin and *decreased* kanamycin tolerance. In contrast, exposure to Roundup caused increased *susceptibility* towards chloramphenicol and tetracycline and increased *tolerance* towards kanamycin and ciprofloxacin. Roundup did not have an effect on ampicillin tolerance in *S. typhimurium*. In herbicide-supplemented media, when antibiotic tolerance was induced the number of viable bacteria increased to levels comparable to the no chemical treatment.

The antibiotics to which *S. typhimurium* became tolerant have different modes of action and targets. Tetracycline and chloramphenicol inhibit protein synthesis, ampicillin inhibits the cell-wall synthesis (Rosner, 1985) and ciprofloxacin inhibits DNA gyrase (Cho *et al.*, 1995). This suggests that the pathway(s) by which the herbicides cause tolerance is not specific to each antibiotic. Rather it may be a more general pathway that renders tolerance to many antibiotics. One pathway that may lead to antibiotic tolerance is through reduced accumulation of the antibiotic within the cell either through increased efflux or reduced influx (Poole, 2004).

Salicylic acid also induces tolerance to antibiotics that have different modes of action and the induced antibiotic response has been linked to changes in the cell membrane. Previous work shows that *E. coli* grown in the presence of salicylic acid has increased levels of the AcrAB efflux pump and decreased porins such as the OmpF porin (Price *et al.*, 2000). The induced antibiotic response seen here may also be due to alterations in the cell membrane permeability and efflux because in *S. typhimurium* the AcrAB-TolC efflux pump complex facilitates the efflux of chloramphenicol, tetracycline, β-lactams and fluoroquinolones (Poole, 2004). Kamba and 2,4-D may up-regulate this efflux pump and decrease the uptake of antibiotics through OmpF. This may decrease the antibiotic concentration within the cell resulting in increased tolerance. In contrast, Roundup may increase a different pump which may remove ciprofloxacin and kanamycin from the cell, leading to increased tolerance.

Unlike the other antibiotics tested, kanamycin is transported through the AcrD efflux pump in *E. coli* (Rosenberg *et al.*, 2000; Eaves *et al.*, 2004). Previous studies have shown that *E. coli* grown in the presence of salicylic acid have increased susceptibility towards kanamycin

(Aumercier *et al.*, 1990) and this may be due to down-regulation of the AcrD efflux pump. *S. typhimurium* also possesses an AcrD efflux pump (Eaves *et al.*, 2004). In order to make a functional efflux pump, AcrD and AcrB need to form a complex with AcrA and TolC (Eaves *et al.*, 2004). It could be that Kamba and 2,4-D increase the number of AcrB which limits the number of AcrA and TolC available to form a complex with AcrD, hence causing the accumulation of kanamycin within the cell. Conversely, Roundup may increase the number of AcrD and/or decrease the number of AcrB, allowing the formation of more functional AcrD pumps which reduce the intracellular concentration of kanamycin and hence increase tolerance.

The concentrations of herbicides that caused an antibiotic response were within the recommended application rates and some were within the Maximum Residue Limits (MRL) set for food and feed. Bacteria are likely to encounter lower concentrations of herbicide residues in the environment compared to the manufacturers recommended application rates. The magnitude of the antibiotic response induced by salicylic acid was dependent on its concentration (Rosner, 1985). To determine if herbicide concentrations within the MRL are sufficient for inducing an antibiotic response, the lowest herbicide concentration that induced an effect was determined. As the herbicide concentration increased, a stronger antibiotic response was induced but after a certain concentration the effect plateaued. The minimum herbicide concentration that induced an antibiotic response varied with each antibiotic and herbicide.

Micro-organisms may encounter high herbicide concentrations immediately after the application of herbicides, but later on they may only be exposed to lower concentrations. This led us to question if the antibiotic response induced by Kamba can be maintained at lower concentrations or in its absence. Here, chloramphenicol, tetracycline, ampicillin and ciprofloxacin tolerance was maintained in the absence of Kamba once the tolerance was induced with Kamba plus antibiotic. Induction of the antibiotic tolerance required the presence of both the antibiotic and Kamba. Pre-treatment with Kamba-only was not capable of inducing tolerance. In addition, the effect of the herbicide was faster than the lethal effect of the antibiotics despite ciprofloxacin, ampicillin and kanamycin having bactericidal properties.

In earlier studies that showed salicylic acid-induced antibiotic tolerance, the bacteria were also incubated in the presence of both the antibiotic and salicylic acid (Rosner, 1985; Aumercier *et al.*, 1990; Berlanga & Vinas, 2000). The antibiotic response may require an initial step that is dependent upon the antibiotic, and once this is triggered the herbicide or salicylic acid is able to up-regulate the specific efflux pumps.

It is perplexing, however, how such different antibiotics all somehow trigger this singular response.

The recommended application rates of the herbicides are above the minimum concentrations necessary to induce an effect. Therefore, bacteria that are exposed to the herbicides may become tolerant to antibiotics and this tolerance may be maintained at lower or nil herbicide concentrations. Furthermore, the tolerance may be maintained for many generations without exposure to the herbicide. Here, chloramphenicol tolerance induced by Kamba was maintained for at least 5 days when colonies were serially re-streaked on luria broth (without renewed exposure to Kamba) and re-tested for chloramphenicol tolerance.

Antibiotics are used in the animal industry (Key & McBride, 2014) and the use of animal farm slurry on crop farms as a fertilizer (Duffy, 2003) can create a breeding ground ideal for the development of herbicide-induced antibiotic tolerance in bacteria. Subsequently these bacteria may be transferred to humans and animals. The development of such tolerant strains can make treatment of disease-causing bacteria difficult. Many of the antibiotics used in the animal industry are similar to those used to treat humans or may be used for therapies of non-infectious diseases at low doses for long term, e.g., tetracycline (Heinemann *et al.*, 2000; Key & McBride, 2014). The level of tolerance provided by the herbicides for some of the antibiotics were close to the tolerances seen in *S. typhimurium* strains that may be untreatable (Hassing *et al.*, 2013). *S. typhimurium* is one of the common causes of bacterial food-borne illnesses that result in numerous hospitalizations (Brunelle *et al.*, 2013); hence, the tolerance induced by the herbicides could compromise treatment.

Herbicides and antibiotics are known to contaminate water reservoirs through run-offs (Davis *et al.*, 2006; Shipitalo *et al.*, 2008). Dicamba and 2,4-D residues have been found in drinking

water in the United States (Donald & Cessna, 2007). Hence, herbicide residues in water may affect the antibiotic response of bacteria. Here, *S. typhimurium* incubated in water contaminated with Kamba and chloramphenicol did not show increased chloramphenicol tolerance. It is unknown as to why chloramphenicol tolerance was not induced in water. It may be due to the lack of nutrients in water or due to density-dependent growth inhibition because of the large starting titre.

Development of glyphosate-tolerant weeds has reduced the effectiveness of glyphosate as an herbicide and as a consequence, farmers are mixing together other herbicides that have different modes of action for effective weed control (Benbrook, 2012). Bacteria are therefore likely to encounter a number of herbicides in the environment. We found that when herbicides were combined, both the herbicides played a role on the antibiotic response. The elicited response was an intermediate response compared to the responses induced by the herbicides individually.

Glyphosate (Benbrook, 2012), dicamba (Behrens *et al.*, 2007) and 2,4-D (Munro *et al.*, 1992) have long been considered to be three of the safer herbicides in terms of human health. However, this study confirms that Kamba, 2,4-D and Roundup induce an antibiotic response in *S. typhimurium* that could potentially affect human health indirectly by increasing the failure rate of treating infections. The novel findings of this research will challenge the current herbicide practices and risk assessment standards, none of which consider these effects. In order to minimize the effects on non-target organisms a more prudent use of antibiotics and herbicides is necessary.

Herbicides are a useful tool in weed management but at the same time they may have adverse effects on non-target organisms. The aim of this study was to increase knowledge about the impact of commercial herbicide formulations on bacteria, with the main focus being on an antibiotic tolerance response to sub-lethal exposures. Due to the increased release of herbicides into the environment it is vital that we understand any unintentional effects they may have on non-target organisms. Although there has been speculation about the selection of antibiotic tolerant strains by biocides (Poole, 2004), most of the studies on the effects of

herbicides on bacteria are limited to toxicity (Busse *et al.*, 2001; Hillaker & Botsford, 2004). Prior to this work, there was no knowledge about the effects commercial formulations of glyphosate, 2,4-D and dicamba have on the antibiotic response in bacteria.

Future Work

Most of the work described here has provided the first evidence of herbicide-induced antibiotic response in *S. typhimurium* and can be used as a foundation for future studies. The research described here could be developed and expanded upon in a number of ways. The current study showed that Kamba-induced antibiotic tolerance can be maintained in its absence. One way to expand on this research would be to determine if the antibiotic tolerance induced by 2,4-D and Roundup can also be maintained in the absence of the herbicide.

Another way to expand on this research is to investigate if there are strain-specific effects that might differ between laboratory strains. The current study has already revealed differences in ampicillin tolerance between *E. coli* (B. Kurenbach and J.A. Heinemann, personal communication) and *S. typhimurium*. Strains that are known to be tolerant to antibiotics and are difficult to treat can also be tested. The increased tolerance caused by the herbicides may provide protection against antibiotics allowing bacterial survival. The ability to stay viable at higher antibiotic concentrations could give bacteria a selective advantage as they may be able to develop genotypic resistance through spontaneous mutations (Berlanga & Vinas, 2000). Previous work has shown that a strain of *Staphylococcus aureus* that is normally susceptible to ciprofloxacin, becomes tolerant in the presence of salicylic acid due to an increased mutation frequency (Gustafson *et al.*, 1999).

Salicylic acid is known to be an efficient inducer of multiple drug tolerance as its presence has been shown to induce tolerance to several drugs simultaneously in *E. coli* (Rosner, 1985). In this study, the effects of multiple herbicides were tested on individual antibiotics. The reverse combination can also be tested (the effect of individual herbicides on tolerance towards multiple antibiotics).

All the experiments conducted here used commercial formulations of herbicides that contain other surfactants apart from the active ingredient. It is not known if the effect seen here is solely due to the active ingredient or if the surfactants also played a role. In order to distinguish between the two, the active ingredients that are in a pure form have to be tested. This will allow us to determine if the active ingredients are capable of inducing an antibiotic response. This work is currently being undertaken and initial results suggest that the active ingredients also affect antibiotic tolerance in similar patterns to the commercial formulations (P. Gibson and J.A. Heinemann, personal communication).

While the effects seen here are consistent with changes in efflux pumps and porins, the exact pathways used by the herbicides remain to be identified. There are a number of ways that can help identify these pathways. Efflux pump inhibitors have been one of the tools used to show that salicylic acid induced ciprofloxacin tolerance in *S. typhimurium* is due to the induction of the *acrAB* operon (Hartog *et al.*, 2010). Phe-Arg-β-naphthlamide (PAβN) is a broad spectrum efflux pump inhibitor in a number of bacteria (Sáenz *et al.*, 2004). *S. typhimurium* that is grown in the presence of PAβN can help confirm that herbicide-induced antibiotic tolerance is due to the up-regulation of efflux pumps. The up-regulation of specific operons by the herbicides can also be identified using strains carrying the operon fused to *lacZ*. Similar to the methods used by Rosner *et al.* (1991), the β-Galactosidase activity can be used to determine the level of expression of the fusion genes (Rosner *et al.*, 1991). Currently, this method is being used to determine if Kamba induces the *soxRS* regulon and initial results show that Kamba is an inducer of this regulon although not as strong as paraquat (C.F. Amabile-Cuevas, personal communication).

To determine the exact genes that are transcribed in tolerant bacteria, quantitative reverse transcription polymerase chain reaction (RT-PCR) can be used. Previous studies have used this method to monitor mRNA levels of particular genes like *acrAB* and *micF* that are involved in ciprofloxacin tolerance in the presence of salicylic acid (Hartog *et al.*, 2010). This method will help identify the specific levels of mRNA molecules in antibiotic tolerant bacteria. Once the genes affected by the herbicides are identified, bioinformatics tools would also be worthwhile to determine other strains of bacteria that may also be affected by the herbicides. A BLAST

search of the gene sequences will help recognize other strains with similar gene sequences that code for efflux pumps or porins.

Other experiments that could be done in the future include studying the changes in gut flora of animals exposed to herbicides and antibiotics. Gut bacteria in farm animals that are exposed to certain antibiotics can develop tolerance, for example chickens that were fed tetracycline-supplemented feed, almost all of their gut flora consisted of tetracycline tolerant organisms within one week (Levy *et al.*, 1976). Similarly, herbicide residues present in hay or grass may accumulate in an animal's gut and under the right conditions could induce antibiotic tolerance. Finally, microbes in rumens may react differently than microbes in other parts of the digestive system.

Similar studies could be performed for the gut flora of insects such as honey bees. In the U.S., beekeepers routinely use antibiotics to treat and protect bees from disease (Tian *et al.*, 2012a). Bees may be exposed to herbicides while plants are being treated with herbicides or when bees feed on nectar or pollen from flowering plants that have been treated with herbicides. Future experiments may be conducted to determine if bees exposed to herbicides and antibiotics cause a shift in the gut flora toward tolerant bacteria and this may reveal the potential environments where herbicides affect non-target organisms.

In the presence of antibiotics, the herbicides Kamba, 2,4-D and Roundup induce an antibiotic response in *S. typhimurium* at concentrations within the recommended application rates. The induced tolerance has been shown to persist in the absence of the herbicide. The fact that Roundup, which is not structurally related to salicylic acid, induced an antibiotic response which indicates that the response may not only be due to the chemical structure but could also be a general response to chemical stress. The mechanisms by which the herbicides induce the antibiotic response are unknown, but may include changes in the efflux pumps, porins or other mechanisms.

The tolerance provided by the herbicides can be potentially important for health care in humans and animals because it may lead to treatment failure. The ability to assess the risks of introducing herbicides into the environment first relies on a solid understanding of their effects

not only on plants but also on non-target organisms like bacteria. There is clearly much work to be done in this area and important issues that need to be addressed in order to reduce the adverse effects of herbicides on non-target organisms.

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